DESIGN, SYNTHESIS AND METABOLISM OF ARODYN ANALOGS

BY

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DESIGN, SYNTHESIS AND METABOLISM OF ARODYN ANALOGS

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ABSTRACT

Our research focuses on the development of kappa opioid receptor (KOR) antagonists. KOR antagonists have a variety of possible therapeutic applications; these compounds have shown anxiolytic activity, anti-depressive activity, and have potential uses in the treatment of opioid and cocaine addiction. Dynorphin A (Dyn A) is an endogenous agonist at KOR. Dyn A has been used as a lead for the development of KOR antagonists including arodyn, an acetylated Dyn A analog identified in our laboratory. Arodyn is a potent and selective KOR antagonist that has shown promise in the treatment of stress-induced relapse of cocaine seeking behavior. However, arodyn is rapidly metabolized in rat brain homogenate and slices. The objective of this research was to synthesize arodyn analogs with greater metabolic stability and to test these analogs in metabolism studies. Previous research in our laboratory had identified the sites of cleavage in arodyn and these sites were targeted for modification. Analogs with extended sequences, N-methylarginine replacements and reduced amide bond stabilizations were synthesized. In metabolism studies in washings from rat brain slices, several analogs were identified that showed increased stability over arodyn.

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## ABBREVIATIONS

Abbreviations used for amino acids follow the rules of the IUPAC-IUB Joint Commission of Biochemical Nomenclature in *Eur. J. Biochem*. **1984**, *138*, 9-37. Amino Acids are in the L-configuration except where indicated otherwise. Additional abbreviations used in this thesis are as follows:

AC: adenylyl cyclase;

Boc: *tert*-butyloxycarbonyl;

cAMP: cyclic adenosine monophosphate;

CHO: Chinese hamster ovary;

DCM: dichloromethane;

DIEA: *N,N*-diisopropylethylamine;

DMF: *N,N*-dimethylformamide;

Dyn A: dynorphin A;

ESI-MS: electrospray ionization mass spectrometry;

Fmoc: 9-fluorenylmethoxycarbonyl;

GDP: guanosine 5'-diphosphate;

GNTI: guanidinium naltrindole;

GPI: guinea pig ileum;

GPCR: G-protein coupled receptor;

GTP: guanosine-5'-triphosphate;

GTPγS: guanosine-5'-(3-thiotriphosphate);

HOBt: 1-hydroxybenzotriazole;

HPLC: high-performance liquid chromatography;

i.c.v.: intracerebroventricular;

i.p.: intraperitoneal;

i.v.: intravenous;

LH-RH: lutenizing hormone-releasing hormone;

Mtr: 4-Methoxy-2,3,6-trimethylbenzenesulfonyl;

norBNI: nor-binaltorphimine;

NMR: nuclear magnetic resonance;

ORL-1: opioid-receptor like-1;

PAL: peptide amide linker;

PEG: poly(ethylene glycol);

PS: polystyrene;

PyBOP:benzotriazol-1-yl-oxytripyrrolidinophosphonium

hexafluorophosphate;

s.c.: subcutaneous;

SAR: structure-activity relationship;

SEM: standard error of mean;

SPPS: solid-phase peptide synthesis;

TFA: trifluoroacetic acid;

Tic: 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid;

TIPS: triisopropylsilane;

TLC: thin layer chromatography.

# 1 Introduction

The objective of this research was to synthesize and evaluate the metabolism of new analogs of peptide kappa opioid receptor (KOR) antagonists.

KOR antagonists can be used for the study of KOR receptor function. There is also interest in the development of KOR antagonists as clinical agents. KOR antagonists have been shown to have antidepressant activity in the forced swim test in rats.[1] KOR antagonists can decrease the withdrawal signs in opioid addicted subjects, and therefore also have potential application in the treatment of opioid addiction.[2, 3] KOR antagonists have shown promise as treatments for cocaine dependence by preventing stress related relapse.[4, 5] Studies on mice showed that KOR antagonists can prevent stress-induced relapse of cocaine seeking behavior.[4, 5]

Non-peptide KOR antagonists such as norbinaltorphimine (norBNI) and (3R)-7-Hydroxy-N-[(1*S*)-1-[(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl]-2-methylpropyl]-1,2,3,4-tetrahydro-3-isoquinoline-carboxamide (JDTic) show extremely long duration of action.[6] NorBNI, injected systemically, maintained kappa opioid receptor (KOR) antagonist activity for up to 4 weeks in mice.[7] JDTic has kappa opioid antagonist effects following oral (p.o.) and subcutaneous (s.c.) administration for up to 28 days in rats.[8] The therapeutic utility of drugs displaying such a long duration of action may be limited because potential side-effects or toxicities could also be long lasting.

The dynorphins are endogenous ligands for KOR, and these have been used as lead compounds for the development of KOR selective ligands. Peptides generally have high selectivity and low toxicity[9], though they are often metabolized more

quickly *in vivo* than small molecule ligands as a result of peptidase activity. Because of this, peptides are generally not expected to exhibit as long-lasting activity as small molecules. Furthermore, peptides and small molecules have been shown to bind to different regions of KOR.[10] Thus, peptides and small molecules may provide different information about this receptor, and peptides could have somewhat different activity than small molecules at KOR. Structural modifications to dynorphin A (Dyn A) have led to the development of novel KOR agonist and antagonist ligands.

Arodyn (Ac[Phe$^{1,2,3}$,Arg$^{4}$,D-Ala$^{8}$]dynorphin A-(1-11)$NH_2$) is a selective and potent KOR receptor antagonist that was developed from a 1-11 fragment of the endogenous KOR agonist Dyn A[11] The design and synthesis of Dyn A analogs has been a focus of the Aldrich laboratory research for several years. Replacement of the "message" sequence of [D-Ala$^{8}$]Dyn A(1-11)$NH_2$ with a μ opioid receptor antagonist followed by structural modifications using a combinatorial library resulted in the discovery of arodyn.[11] A comparison of dynorphin A and arodyn structures is shown in Figure 1-1.Arodyn exhibits high KOR selectivity and antagonist potency.[11]

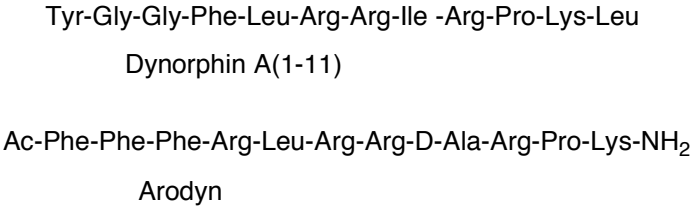


**Figure 1-1: Comparison of dynorphin A(1-11) and arodyn structures.**

Arodyn was evaluated in a tail withdrawal assay and a cocaine conditioned place preference assay.[4] Several small molecule KOR antagonists and arodyn were tested in the tail withdrawal assay against the KOR agonist U50,488. These assays showed that withdrawal latencies were reduced for 1-3 days for arodyn pretreated mice compared to saline-pretreated mice. The small molecule KOR antagonists norBNI and JDTic had much longer lasting antagonist activity; tail withdrawal latencies were reduced for at least 7 days.[4]

A conditioned place preference assay was carried out for arodyn.[4] Mice were first treated with cocaine, associated with only one compartment in the apparatus. The mice then showed a preference for the cocaine-associated compartment. Cocaine was removed for three weeks and the place preference became extinct. Following extinction, mice were subjected to a forced swim-stress protocol. Those mice pretreated with arodyn did not relapse into drug seeking behavior, while mice in the control group exhibited reinstatement of the cocaine place preference.[4]

Metabolic studies in the Aldrich laboratory showed arodyn to be rapidly metabolized in rat blood or brain homogenate, with complete disappearance of the parent peptide in under 15 minutes.[12] These studies identified potential sites of metabolism.

Arodyn has shown promise in *in vivo* assays in preventing reinstatement of cocaine abuse. Its short half-life, though, limits its potential as a therapeutically useful drug. More metabolically stable analogs could lead to the development of a drug for the treatment of cocaine addiction. In addition, stable analogs may provide

insight into whether a longer lasting metabolite is responsible for arodyn's long-lasting activity.

## *1.1 Objectives and Hypotheses*

By modification of potential cleavage sites we can increase the metabolic stability of arodyn. The objective of the research presented in this thesis is to identify the modifications to arodyn that increase the stability of the peptide. In addition, the selectivity and potency of arodyn should be maintained in the new analogs.

Previous investigation into the structure-activity relationships for arodyn[13] identified the arginine residues as the most important for binding – replacement of these residues with alanine resulted in the largest decreases in affinity for KOR. Thus, these side chains should be retained in new analogs.

The potential cleavage sites identified in an earlier metabolism study[12] are clear targets for stabilization. Since these potential cleavage sites were next to arginines it is necessary to make modifications that stabilize the peptide bond while maintaining the arginine side chain functionality. Thus, backbone modifications (N-methylarginine substitutions and reduction of the peptide bond) have been chosen, in addition to C-terminal extended analogs.

Metabolism studies using washings from rat brain slices were performed to assess which analogs, if any, had increased stability over arodyn.

## *1.2 Research*

### 1.2.1 Design and Synthesis of Arodyn Analogs

A series of four reduced amide bond analogs, a series four of N-methylarginine analogs and two extended analogs were designed and synthesized. In to determine which modifications are most effective at slowing metabolism, only one modification was made per analog.

Substitution of each arginine with an N-methylarginine maintains the side-chain functionality while stabilizing the peptide bond to peptidase metabolism. The more sterically hindered N-methyl peptide bond should hinder peptidase cleavage at this site.[14] Each arginine was replaced in separate analogs.

Reduced amide bonds replace the peptide bonds with amine functionalities. This, like the α-N-Me-Arg replacement, maintains the original side chains. The cleavage at this site should be completely blocked, as there is no longer a peptide bond for a peptidase to attack.[14] The reduced amide bond was introduced at the N-terminus of each arginine residue in separate analogs.

Extended analogs are attractive because they are simple modifications which may change the rate of breakdown by peptidases. It has been observed that rates of peptidase breakdown of dynorphins vary by the size of the dynorphin fragment.[15] Thus, it is possible that simply extending the peptide sequence of arodyn may alter metabolism.

Since extending a peptide sequence involves standardized coupling and deblocking procedures to add each additional amino acid. These modifications are easily implemented.

### 1.2.2 Metabolism Studies

Metabolism studies were done in order to identify the modifications that most hindered metabolism. Seven analogs were tested in the initial metabolism studies. The metabolism of these analogs was followed for 60 minutes and analyzed using MALDI-MS (matrix assisted laser desorption/ionization mass spectrometry). Several analogs were identified that were more stable than arodyn (lasting up to 30 or even 60 minutes in these studies, compared to less than 10 minutes for arodyn).

## *1.3 Significance*

There are nearly 5.3 million cocaine users in the U.S.[16] Currently there are no medications available for the treatment of cocaine addiction.[17] KOR antagonists have shown potential as therapeutics for stress-induced relapse of cocaine addiction. Stress can enhance the rewarding properties of drugs of abuse and has been shown to induce relapse leading to drug abuse and to potentiate drug-seeking behavior.[18] KOR antagonists have been shown to reduce aversive behavior in mouse stress tests.[19]

Arodyn showed potential as a cocaine addiction treatment in conditioned place preference tests in mice. However, arodyn is rapidly degraded in rat whole blood and brain homogenate due to endopeptidase metabolism, with complete

disappearance of the parent peptide in under 15 minutes.[12] In order to produce a therapeutically useful drug, the stability of this peptide would have to be increased.

New analogs could lead to the development of stable compounds and a therapeutically useful drug. Development of stable analogs might also give insight into whether arodyn's long-lasting activity is due to an active metabolite.

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# 2 Literature Review

## 2.1 Opioid Receptors

Opium, derived from the latex of the opium poppy, *Papaver somniferum*, has been used as a medicinal agent for centuries.[1] The major active ingredient, morphine, was first isolated in 1805.[2] Modifications of the structure of morphine have produced other pharmacologically active compounds, such as heroin. However, it was not until the 20th century that opioid receptors were discovered to be the site of action of these compounds.[3]

### 2.1.1 Classification and Endogenous Ligands

Based on behavioral studies of chronic spinal dogs, Martin[4, 5] posited the existence of three types of opioid receptors. These receptors were labeled according the drugs used in the study - mu (μ) for morphine and kappa (κ) for ketacyclazocine. A new type of opioid receptors, delta (δ) opioid receptors, was proposed in 1977 by Kosterlitz and coworkers based on studies with enkephalins in the mouse vas deferens (MVD).[6] The μ opioid receptor (MOR), κ opioid receptor (KOR), and δ opioid receptor (DOR) have all been cloned.[7-9] In addition, a new type of opioid-like receptor was discovered and cloned.[10] This receptor does not bind classical opioid agonists or antagonists with high affinity and, therefore, has been called the opioid-receptor like (ORL-1) receptor.

In the 1970s, Hughes *et al.*[11] isolated two pentapeptides from mammalian brain tissue - Leu-enkephalin and Met-enkephalin. These peptides showed morphine-like activity and were shown to be opioid agonists in the guinea pig ileum (GPI) and

MVD assays. The name "enkephalin" was given to these compounds after kaphale - the Greek work for "from the head". Later, other endogenous opioid peptides were discovered: β-endorphin,[12] the dynorphins[13] and, recently, the endomorphins.[14] Table 2-1 summarizes the mammalian endogenous ligands for MOR, KOR and DOR along with their receptor selectivities.

Endogenous ligands for KOR arise from the precursor protein prodynorphin. When prodynorphin is cleaved during processing, multiple active peptides are released: dynorphin A, dynorphin B, α– and β-neoendorphin.[15]

**Table 2-1: Endogenous Opioid Ligands**

| Peptide | Amino Acid Sequence | Selectivity[1] |
|---|---|---|
| Leu-enkephalin | Y-G-G-F-L | δ>μ>>κ |
| Met-enkephalin | Y-G-G-F-M | δ>μ>>κ |
| β-Endorphin | Y-G-G-F-M-T-S-E-K-S-Q-T-P-L-V-T-L-F-K-N-A-I-I-K-N-Y-A-Y-K-G-E | μ≅δ |
| Dynorphin A | Y-G-G-F-L-R-R-I-R-P-K-L-K-W-D-N-Q | κ>μ>δ |
| Dynorphin B | Y-G-G-F-L-R-R-N-F-K-V-V-T | κ>μ>δ |
| α-Neoendorphin | Y-G-G-F-L-R-K-Y-P-K | κ>δ,μ |
| β-Neoendorphin | Y-G-G-F-L-R-K-Y-P | κ>δ,μ |
| Endomorphin-1 | Y-P-W-F-$NH_2$ | μ>>κ, δ |
| Endomorphin-2 | Y-P-F-F-$NH_2$ | μ>>κ, δ |
| Nociceptin/orphanin FQ | F-G-G-F-T-G-A-R-K-S-A-R-K-L-A-N-Q | ORL1 |

## 2.1.2 Pharmacology

Opioid receptors are part of the rhodopsin subclass of G-protein coupled receptors (GPCRs). GPCRs have characteristic seven helical transmembrane regions,

an extracellular N-terminus, and an intracellular C-terminus.[16] A diagram of a GPCR is shown in Figure 2-1.

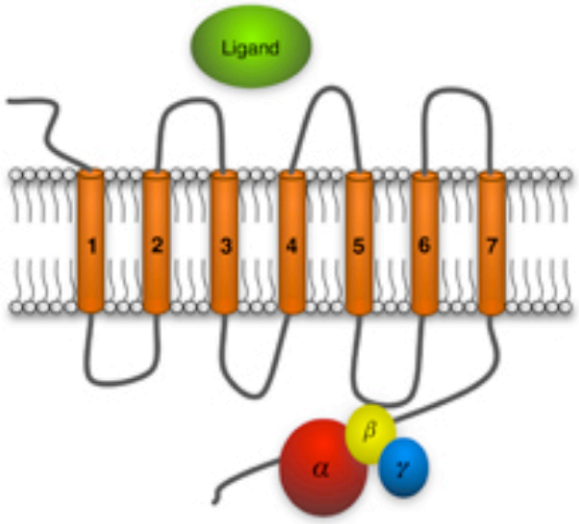


**Figure 2-1: G-protein coupled receptor(from berkely.edu[17])**

Opioid receptors are coupled to the $G_i/G_o$ family of G-proteins at their intracellular domain. All G-proteins are composed of α, β and γ subunits. After agonist binding the receptor changes conformation which promotes the release of guanosine 5'-diphophate (GDP) from the α subunit. The α subunit then binds gunaosine 5'-triphophate (GTP). The G-protein then dissociates from the receptor, and the α subunit is separated from the βγ dimer. The α subunit and βγ dimer can then associate with other molecules inside the cell to modulate biological functions.[18, 19] When GTP is hydrolyzed to GDP by the α subunit, the G-protein can then reassemble and reassociate with the receptor.

$G_i$ alpha (or $G_o$ alpha) is a subunit that inhibits the production of cAMP from ATP, which results in decreased activity of cAMP-dependent protein kinase. This results in lowered neurotransmitter release in neurons.[20]

### 2.1.3 Kappa Opioid Receptors

Drugs affecting the μ opioid receptor (such as morphine and heroin) are associated with analgesia, but also with the side effects respiratory depression, constipation, euphoria and physical dependence. The δ opioid receptor can also mediate analgesic effects, but can have the side-effect of seizures.[21]

KOR agonists have been shown to be analgesics without the μ-associated adverse effects;[22] The effectiveness of KOR agonists depends on the pain type. For some types of pain (e.g. thermal) KOR agonists are not as effective as MOR agonists.[1] Recently, studies have established that KOR agonists can produce analgesia peripherally both in somatic and visceral pain models, particularly in certain conditions involving inflammation.[23] However, KOR agonists have been reported to cause side effects such as dysphoria.[24, 25] Recently, some KOR agonists were reported to have the potential to be used as peripheral analgesics without central nervous system (CNS) side effects,[26] and, therefore, there has been an interest in developing peripheral KOR agonists[1] for the treatment of pain.

KOR agonism has a wide variety of effects beyond analgesia. Activation of KOR antagonizes many of the effects of MOR.[27] Also, kappa agonists may be neuroprotective against hypoxia/ischemia.[28] KOR agonists, especially the arylacetamide series of agonists, have been shown to have anticonvulsant and neuroprotective properties, and may have potential to treat epilepsy, stroke, or trauma resulting from brain or spinal cord injury.[29] KOR agonists have also been shown to cause the down-regulation of HIV expression in microglia[30] and CD4+ lymphocytes.[31]

KOR antagonists can be used for the study of KOR function. Currently, there is also increased interest in the development of KOR antagonists as clinical agents. KOR antagonists have been shown to have antidepressant activity in the forced swim test in rats[32] and anti-anxiety activity in rats. [33] KOR antagonists can decrease the withdrawal signs in opioid addicted subjects, and therefore also have potential application in the treatment of opioid addiction.[34, 35]

In addition, both kappa agonists and antagonists have shown potential as treatments for cocaine dependence, but by different mechanisms as will be described below.

### 2.1.4 Nonpeptide KOR Antagonists

Nor-binaltorphimine (BNI) (Figure 2-2) was an early small molecule KOR antagonist reported in 1987.[36] NorBNI has high potency and selectivity for KOR. Structure-activity relationship (SAR) studies of the δ receptor antagonist naltrindole

led to the discovery of a highly selective κ antagonist, 5'-guanidinium naltrindole (GNTI, Figure 2-3).[37]

The small molecule KOR antagonist JDTic (Figure 2-4) was reported in 2001 by Thomas *et al.*[38] It was later shown to suppress stress induced-reinstatement of cocaine-seeking behavior in rats.[39]

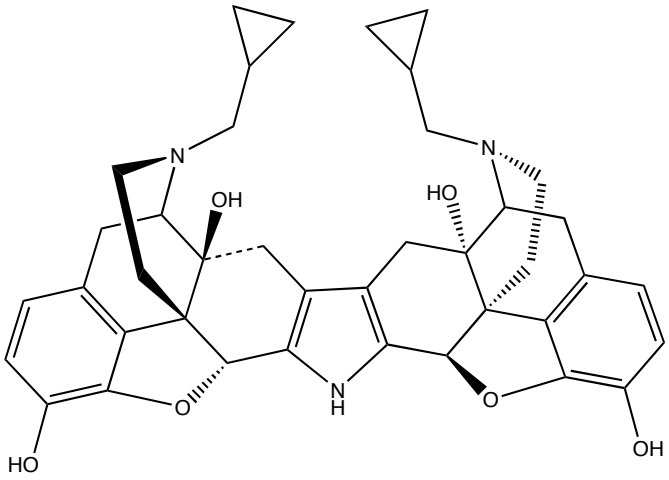


**Figure 2-2: NorBNI**

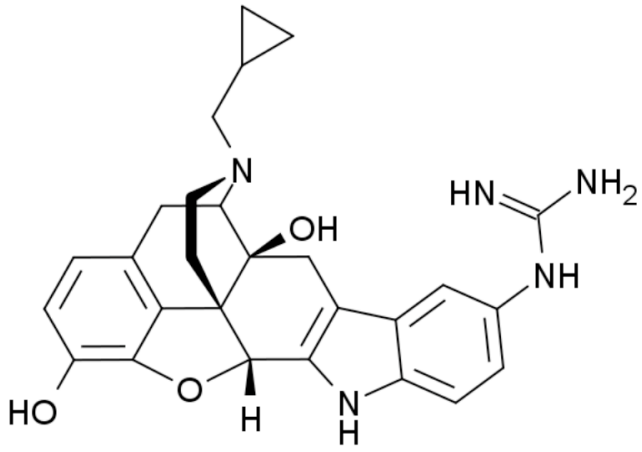


**Figure 2-3: GNTI**

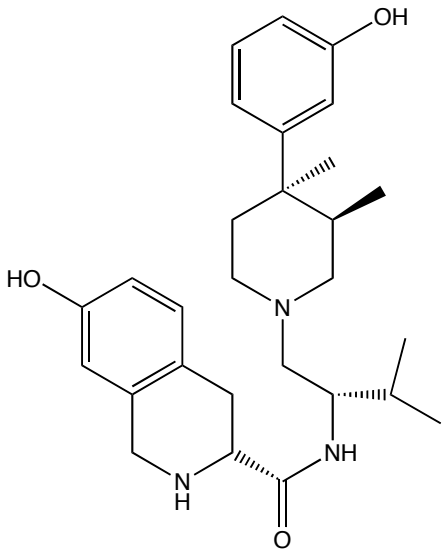


**Figure 2-4: JDTic**

### 2.1.5 Cocaine Abuse

Cocaine is a psychostimulant found in the leaves of the coca plant. There are nearly 5.3 million cocaine users in the U.S.[40] Between 5-6% of new users become dependent within two years of their first use of cocaine.[40] Given roughly 1.2 million new users in 2001, an estimated $86,400,000 per year would be required to treat just new cases.[40] Cocaine use can result in severe paranoia, mood disturbances, hallucinations, seizures and fatal overdose.[41]

In addition to the private toll of addiction, there are public costs in terms of public health and law enforcement. Thirty-seven percent of state law enforcement agencies in the United States cited cocaine as the greatest drug threat, according to

the 2003 National Drug Threat Survey.[41] Currently there are no medications available for the treatment of cocaine addiction.[42]

Cocaine acts by blocking dopamine uptake by the dopamine transporter (DAT), effectively increasing dopamine signaling, which is thought to be associated with the rewarding effects of the drug.[43, 44] Thus, some medications under development act as "agonist substitutes" for cocaine and target dopamine receptors or DAT.[45, 46] However, DAT gene knockout mice still self-adminster cocaine, and studies implicate serotonin and norepinephrine transporters in the pharmacological effects of psychostimulants.[47] Therefore, disruption of dopamine or DAT activity may not be sufficient to treat cocaine addiction.

Thus, the kappa opioid receptor presents a novel target for the treatment of cocaine addiction.

### 2.1.6 Drug Abuse Treatments Targeting KOR

Both agonists and antagonists of KOR have shown promise in treating cocaine dependence. They appear to work via different mechanisms, making each possible agents for the treatment of addiction.

#### 2.1.6.1 KOR Agonists

The endogenous KOR ligands, the dynorphins, have been found to modulate both pain and addiction.[48] Kappa opioid receptors are located in proximity to dopamine receptors in the nucleus accumbens which may be why KOR agonists affects dopamine signaling.[49] KOR activation is known to inhibit dopamine (DA)

release[50] and is connected to cocaine aversion.[51] Agonism of KOR also affects DAT uptake of DA.[52] Administration of E-2078, a dynorphin A analog, was shown to decrease DA release.[53] When dynorphin A is infused into a mouse brain, it suppresses cocaine-induced release of DA and reward (as measured by a condition place preference assay).[54] Further evidence that kappa agonism blocks cocaine's rewarding effects is that the kappa agonists U50,488 and spiradoline produce dose-related acute decreases in cocaine self-administration in rats.[55]

However, while KOR agonists can suppress cocaine reward and self-administration over short courses of treatment, repeated treatment actually increases cocaine craving. Repeated treatment results in a lowering of DAT activity (thus increasing dopamine signaling) without changing the total levels of expression of DAT protein.[41] Stress and depression are common factors in relapse of cocaine abuse and KOR agonism is involved in the stress response (specifically mediating the dysphoric feelings associated with stress[56]). Conversely, KOR antagonists exhibit antidepressant activity.[32]

Thus, while KOR agonists may be suitable for short-term suppression of cocaine reward and craving, they do not appear suitable for longer-term prevention of relapse.

### 2.1.6.2 Kappa Antagonists

KOR antagonists have also shown potential for treatment of addiction, particularly in prevention of stress-induced relapse of cocaine-seeking behavior.[37, 39,

[57] This may be mediated by an entirely different mechanism than KOR agonists. KOR antagonists prevent the dysphoric feelings associated with stress.[56]

The non-peptide KOR anatagonists, GNTI, norBNI and JDTic all have unusually long-lasting pharmacological effects. In mice, KOR antagonism lasts for up to 3 weeks after a single injection of norBNI.[58] Antagonism due to JDTic is similarly long, lasting at least 10-14 days.[59, 60] The mechanism for these effects is not entirely clear, although theories have been proposed. Bruchas *et al.* found that norBNI did not downregulate κ opioid receptors and, as expected, it did not bind to the receptor covalently. They proposed that activation of c-Jun N-terminal kinase (JNK) phosphorylation is involved, although both the KOR antagonist norBNI and KOR agonist U50,488 produced JNK activation.[59] Thus, JNK activation alone is not sufficient to produce long-lasting antagonism.

In results by Aldrich and McLaughlin,[61] binding assays following treatment with arodyn or the small molecule antagonists, either *in vitro* or *in vivo*, indicated that arodyn did not affect receptor density, while norBNI and JDTic lowered receptor density. They propose that long-lasting activity of these small molecules may be attributed to a depot effect.

The long-lasting pharmacological effects of the small molecule selective KOR antagonists pose concerns because of potential toxicity issues. If the antagonistic effects last for several weeks, so might any toxic effects. Thus, there is interest in developing new antagonists that might not have as long-lasting activity.

## 2.2 Peptides as Therapeutics

Peptides often have high activity, high specificity and low toxicity.[62] Eli Lilly's synthetic parathyroid hormone has a dosage of only 20 micrograms a day.[62] Peptides have been marketed as successful drug. The synthetic hormone insulin lispro brings in $1.1 billion a year for Eli Lilly.[62] Daptomycin is a lipopeptide antibiotic, and ziconotide, a synthetic version of a sea snail venom used to treat chronic pain, is also on the market.[62]

There are several potential drawbacks to peptides as drugs, including low oral availability, so another route of administration (primarily injection) is often used. In addition, peptides are often metabolically unstable and are rapidly cleared from the body, have difficulty crossing membranes, and can be expensive to synthesize. However, many of these drawbacks can be overcome. Slow release injectable formulations and nasal sprays of current peptide drugs are on the market (injectable leuprolide formulations can last for months). Metabolic instability can be addressed through structural modifications to slow peptidase cleavage. The ability to cross membranes can also be improved; for example, the dynorphin A analog E-2078 (NMeTyr-Gly-Gly-Phe-Leu-Arg-NMeArg-D-Leu-$NHC_2H_5$) was found to cross the blood brain barrier (BBB), and further in-depth studies reported that this peptide was transported by absorptive-mediated endocytosis.[63] Finally, although peptides are costly on weight basis to synthesize, they can also have high potencies and thus less is needed per dose.

## *2.3 Discovery of Arodyn*

### 2.3.1 Dynorphins

Dynorphin A (Dyn A) is the most studied endogenous ligand for the κ opioid receptor. Dyn A (Figure 2-5) was first isolated from porcine pituitary gland.[64] Dyn A is implicated in motor dysfunction, cardiovascular effects, inflammatory response, feeding behaviors, immunomodulation and stress,[56, 65, 66] although some of these effects are not mediated by opioid receptors. Dyn A(1-13) has been shown to suppress opiate withdrawal and tolerance when administered to morphine-dependent mice.[67]


**Figure 2-5: Dynorphin A**

The structure-activity relationships of dynorphin A have been explored.[1, 68, 69] The N-terminal sequence of this peptide is Tyr-Gly-Gly-Phe, the same as for most mammalian opioid peptides; the C-terminal sequence is different from other endogenous mammalian opioid peptides. Goldstein and coworkers proposed the "message-address" hypothesis for Dyn A[70] (an extension of the original concept posited by Schwyzer[71]), where the N-terminal sequence is the "message" that is responsible for activation of opioid receptors, while the C-terminal sequence is the "address" that is responsible for KOR affinity.[70] Thus, the Tyr-Gly-Gly-Phe sequence is general for the entire opioid family, while the C-terminal sequences of endogenous

peptides vary according to the receptor type for which the peptide preferentially interacts.

Dynorphin A has been used as a starting point for the development of KOR antagonists. These include dynantin, where the N-terminal tyrosine was replaced with a des-amino tyrosine analog. Dynantin has high affinity, selectivity and potency at KOR.[72] Another analog, $Pro^3$Dyn A(1-11)$NH_2$ has very high selectivity, but the antagonist potency is weak.[73]

Our laboratory has developed several promising Dyn A analogs that function as KOR antagonists. The cyclic analog [$N^{\alpha}$-benzyl$Tyr^1$,*cyclo*(D-$Asp^5$,$Dap^8$)]-dynorphin A(1-11)$NH_2$ exhibits nanomolar affinity and high selectivity for KOR.[74]

Chimeric analogs have been developed which combine the "address" sequence of Dyn A with a small peptide with known antagonist activity. In our laboratory, a chimeric Dyn A(1-11) analog, JVA901, was developed.[75] The pentapeptide Boc-Tyr-Lys-Trp-Trp-$NH_2$ was known to be a selective but weak KOR antagonist.[75] An N-terminal acetylated tetrapeptide derivative was combined with the "address" sequence (resides 5-11) of [D-$Ala^8$]Dyn A(1-11)$NH_2$ to produce JVA901. This peptide showed improved KOR affinity and selectivity compared to the starting tetrapeptide and demonstrated antagonist activity against Dyn A (1-13)$NH_2$ in the adenylyl cyclase assay.[75]

Replacement of the "message" sequence of [D-$Ala^8$]DynA(1-11)$NH_2$ with a MOR antagonist (Ac-Arg-Phe-Met-Trp-Met-Arg-$NH_2$) followed by structural modifications using a combinatorial library resulted in the discovery of arodyn (Figure 2-6), a

potent and selective KOR antagonist.[76] Arodyn ([Ac-Phe$^{1,2,3}$,Arg$^{4}$,D-Ala$^{8}$]Dyn A-(1-11)$NH_2$), exhibits high KOR affinity and selectivity ($K_i$(KOR) = 10nM, $K_i$ ratio (KOR/MOR/DOR) = 1/174/538) as well as potent antagonist activity ($K_B$ = 2.0 nM in the adenylyl cyclase assay using cloned κ opioid receptors).[76]

Ac-Phe-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-$NH_2$

**Figure 2-6: Arodyn**

### 2.3.2 Tail Withdrawal Assay

Arodyn was compared to the small molecule KOR antagonists norBNI and JDTic in an antinociceptive assay.[57] Mice were pretreated with a KOR antagonist or the control saline, then later given the KOR agonist U50,488 and subjected to the 55 $^{\circ}$C warm water tail wihtdrawal test. Mice pretreated with arodyn (i.c.v.) had significantly shorter tail withdrawal latencies than mice pretreated with saline for up to 3 days after the arodyn dosage (Figure 2-7, the decrease in tail withdrawal latency at 7 days was not statistically significant). Both norBNI and JDTic exhibited extremely long-lasting activity in this assay; animals pretreated with these compounds had significantly lowered tail withdrawal latencies for at least 7 days after pretreatment. Thus, while arodyn has somewhat long-lasting activity (1-3 days), it is not as extremely long lasting as norBNI or JDTic.

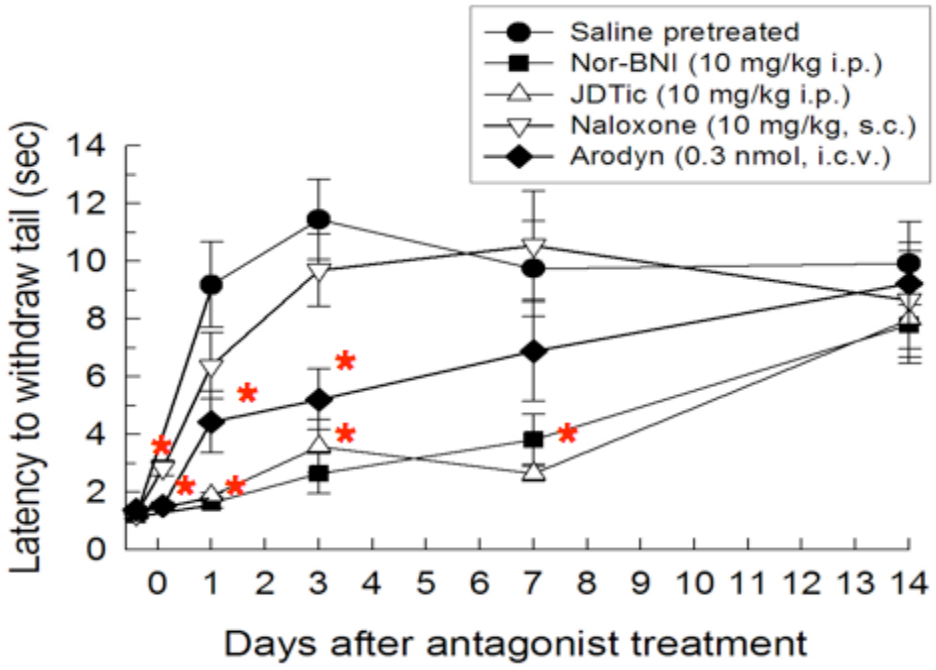


**Figure 2-7: Mouse latency to tail withdrawal from 55°C water: time course of KOR antagonism after a single pretreatment with the drugs listed. * = sig. different ($p < 0.05$) from saline control**[57]

### 2.3.3 Conditioned Place Preference

Arodyn showed promise in preventing stress-related relapse of cocaine seeking behavior in mice (Figure 2-8).[57] Mice were first given cocaine, which was distributed from one compartment. After establishing a conditioned place preference (CPP) for the cocaine-associated compartment, the cocaine was then removed and the CPP was extinguished after 3 weeks. Mice pretreated with arodyn and then subjected to forced swim stress did not show reinstatement of CPP, while the control group did exhibit reinstatement of CPP after the stressor. Reinstatement of CPP could also be produced with a single dose of cocaine after extinction. This effect is not blocked by

arodyn or other KOR antagonists.[57] This supports a role of KOR in stress-induced relapse of cocaine seeking behavior.

The tail withdrawal assay shows that arodyn is a KOR antagonist *in vivo* and the CPP study demonstrated that a peptide KOR antagonist such as arodyn may have potential in the treatment of stress-induced relapse of cocaine abuse.

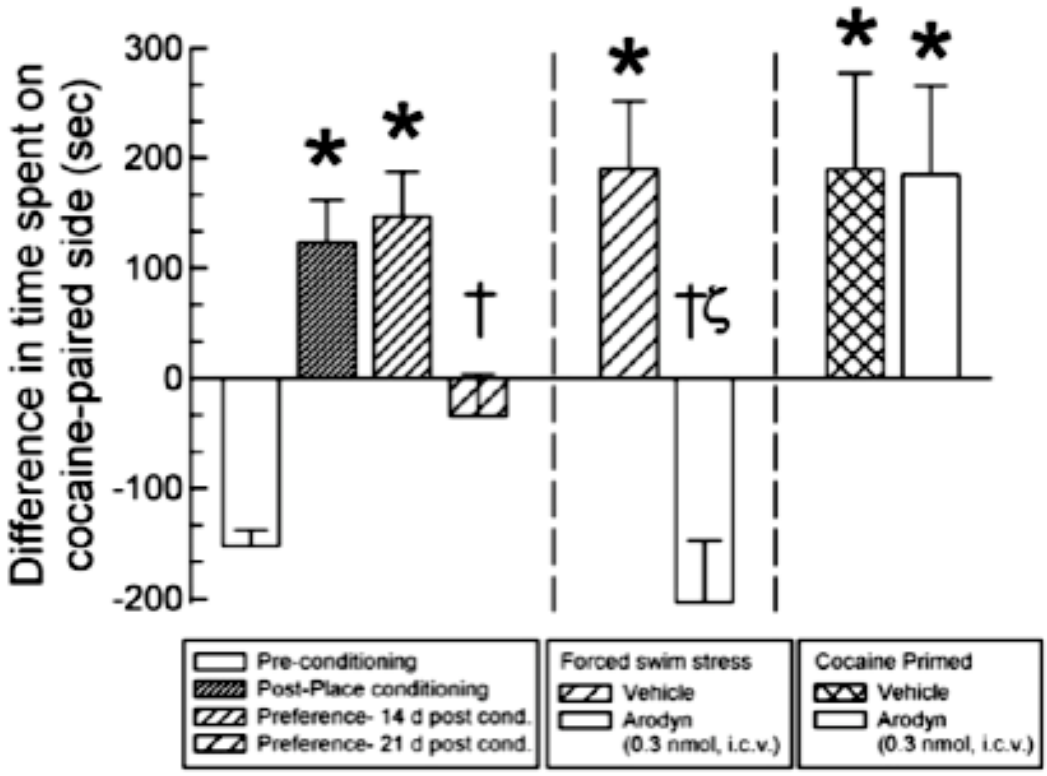


**Figure 2-8: Arodyn (0.3 nmol i.c.v.) blocks stress-induced, but not cocaine primed, reinstatement of cocaine CPP *, †, ζ = sig. different ($p < 0.05$) from preconditioning, postconditioning, and matched vehicle control animals. [57]**

### 2.3.4 Arodyn SAR

The SAR of arodyn was previously investigated;[77] results are shown in Figure 2-9. The SAR of arodyn is unusual in that no particular residues stand out as critical (substitution of some arginines with alanine results in a > 5 fold decrease of affinity for KOR). The SAR also was interesting when compared with that of Dyn A(1-11). Substitution of phenylalanine in positions 1–3 of arodyn causes only a 4-fold decrease in affinity for KOR. Substitution of aromatic residues in positions 1 and 4 of Dyn A(1-13) results in very large decreases in opioid receptor affinity (greater than 500 and 270-fold decreases, respectively).[78] Also, substitution of $Tyr^1$ in Dyn A(1–11)$NH_2$ with Phe results in a 21-fold loss in binding affinity for KOR, [79]while substituting Tyr in place of Phe in position 1 of arodyn results in the much smaller 7-fold decrease in affinity for κ opioid receptors.[77]

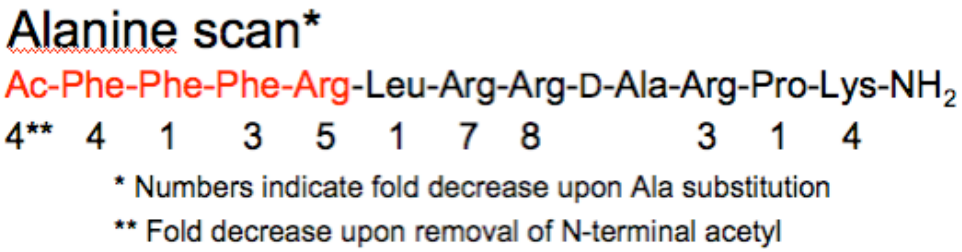


**Figure 2-9: Arodyn SAR[77]**

## *2.4 Metabolism*

Metabolism plays a large role in the pharmacokinetic and pharmaceutical properties of drugs. Most unmodified peptides are rapidly degraded in plasma, which limits their application as therapeutics. In blood, a variety of peptidases (endo- and

exopeptidases) are present that cleave peptides efficiently.[80] Exopeptidases (aminopeptidases, carboxypeptidases, peptidyl di- and tripeptidases) act on the free termini (amino or carboxy) of a peptide chain. Endopeptidases act preferentially in the inner region of a peptide chain away from the amino and carboxy termini.

### 2.4.1 Dynorphin A Metabolism

The metabolism of dynorphin analogs has been analyzed previously by LC-MS (liquid-chromatography mass spectrometry) in brain microdialysis[81,82] and plasma samples.[83] Both ESI[84,85,81] and MALDI (matrix-assisted laser desorption ionization) based MS methods[82,83] have been utilized for the investigation of the metabolism of dynorphin peptides in a variety of biological matrices.

In rat plasma, dynorphin A fragments undergo rapid metabolism ($t_{1/2}$< 5 min) by plasma enzymes.[86] Dynorphin A is cleaved by membrane bound amino- and carboxypeptidases as well as other endogenous enzymes in the central nervous system (CNS).[82] Dyn A(1-13) can release shorter enkephalin sequences after metabolism.[86, 87]

The metabolic fragments observed from Dyn A breakdown depends both on the type of tissue as well as the length of the starting fragment (i.e. Dyn A(1-13) can be metabolized differently than Dyn A(1-11).[80]

Several enzymes involved in the break down of dynorphin have been identified. Thimet-oligopeptidase (EC 3.4.24.15) is a metallo-endopeptidase that has been reported to be involved in the breakdown of a variety of peptides at the Leu-Arg bond, including the dynorphins.[88] Several endopeptidases that cleave dynorphin A at

monobasic and dibasic sites have been investigated.[82, 89-93] These enzymes include dynorphin A converting enzyme and dynorphin A(1-17) processing enzyme. A summary of these enzymes and their cleavage sites is in Table 2-2.

**Table 2-2: Endopeptidase biotransformation of dynorphin A, adapted from Reed *et al* (2003)[82]**

| Primary Fragments Observed | Cleavage Sites | Enzyme | Reference |
|---|---|---|---|
| Dyn A(1-5), Dyn A(6-17) | $Leu^5$-$Arg^6$ | Dynorphin A Converting Enzyme (EC 3.4.22) | Silberring *et al* (1992) [93] |
| Dyn A(1-6), Dyn A(7-17) | $Arg^6$-$Arg^7$ | | |
| Dyn A(1-7), Dyn A(8-17) | $Arg^7$-$Ile^8$ | | |
| Dyn A (1-9) | $Arg^9$-$Pro^{10}$ | Proprotein Convertase 2 (EC 3.4.21) | Day *et al* (1998)[94] |
| Dyn A(1-6), Dyn A(7-17) | $Arg^6$-$Arg^7$ | Dynorphin A Processing Enzyme (EC 3.4.24) | Berman *et al* (1999)[89] |
| Dyn A(1-7), Dyn A(8-17) | $Arg^7$-$Ile^8$ | unidentified | Reed *et al* (2003)[82] |

### 2.4.2 Arodyn Metabolism

Arodyn is acetylated at the N-terminus and amidated at the C-terminus, and thus should be stable to exopeptidase metabolism However, arodyn is susceptible to rapid metabolism by endopeptidases present in whole blood and brain. The half-life in rat plasma was found to be 104±4 minutes, but less than 10 minutes in rat brain homogenate. Studies in the Aldrich laboratory using washings from striatal rat brain slices identified these metabolism sites (Figure 2-10) and followed the time course of the degradation over 30 minutes.[95] MALDI-MS (matrix assisted laster desorption/ionization) was used to identify the metabolites. Table 2-3 lists the metabolites found at each time point along with the conditions - no inhibitors used, the aminopeptidase inhibitor bestatin, a carboxypeptidase inhibitor (CPase), and both

bestatin and the carboxypeptidase inhibitor. The inhibitors were used to prevent exopeptidase metabolism after cleavage by endopeptidases – this would allow for the identification of potential endopeptidase cleavage sites. In all but the last case the parent peptide (*m/z* 1535) disappears before 15 minutes. Peptidase cleavage was found to occur at the N-termini of each arginine residue.[96]

These sites show some similarities to the dynorphin metabolism sites discussed earlier, with cleavages occurring at mono- and dibasic sites. Similar to dynorphin A, arodyn is cleaved at Leu$^5$-Arg$^6$ and Arg$^6$-Arg$^7$ as well as at D-Ala$^8$-Arg$^9$ (D-alanine substitution at the 8 position for isoleucine in Dyn A). In addition, a monobasic cleavage at Phe$^3$-Arg$^4$ is seen.[95]

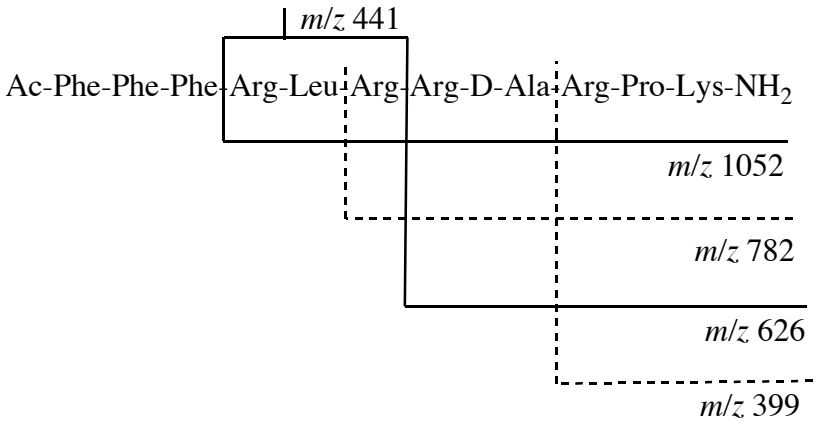


**Figure 2-10: Observed metabolites for arodyn following incubation with wash from striatal rat brain slices.[96]**

**Table 2-3:Mass of arodyn metabolites following incubation with washing from in rat striata slices[96]**

| Time (min) | No Inhibitor | Bestatin | Carboxypeptidase Inhibitor | Bestatin + Carboxypeptidase Inhibitor |
|---|---|---|---|---|
| 0 | 626, 782, 1535 | 441, 1535 | 441, 1535 | 441, 1535 |
| 5 | 399, 441, 626, 1535 | 441, 626, 782, 1052, 1535 | 626, 782, 1535 | 626, 782, 1535 |
| 15 | 399, 441, 626 | 441, 626, 782 | 441, 626 | 626, 782 |
| 30 | 399, 441, 626 | 441, 626, 782 | 626 | 441, 626, 1535 |

### 2.4.3 Strategies for Increasing Metabolic Stability

Modifications to the structures of peptides can increase the biological stability of these compounds. These modifications can be targeted to the potential enzymatic cleavage sites (e.g. N-terminal protection for a peptide susceptible to aminopeptidase metabolism). Some commonly used strategies are described below.

#### 2.4.3.1 Modifications to the N and C Termini

Most peptides with free N- or C- termini are rapidly metabolized in plasma by exopeptidases.[97] A few naturally occurring hormones, including thryotrphin releasing hormone (TRH) and melanocyte stimulating hormone (α-MSH), are stable in plasma.[97] These peptides have modifications g at the N- and C- termini. TRH has an α-pyroglutamyl at the N-terminus and α-prolineamide at the C-terminus that block attack from exopeptidases.[97] α-MSH is protected at the N-terminus by acetylation and at the C-terminus by amidation.[97]

Terminal protection of peptides is a commonly used technique in drug development. In one example, the pentapeptide thymopoietin exhibits an *in vivo* half-

life of 1 min, while the double protected analog was observed to undergo no detectable degradation (for over 30 minutes).[98]

Although protection of the N- and C-termini can stabilize peptides to metabolism by exopeptidases, this generally provides no protection against endopeptidase attack, as demonstrated with arodyn.

### 2.4.3.2 Chirality Changes

Naturally occurring peptides and proteins are made up of L-amino acids and exhibit high stereochemical specificity for interaction with enzymes. Thus, replacing L-amino acids with D-amino acids can improve stability. However, these alterations could change the preferred conformation of the peptide and could affect the biological activity.[97]

### 2.4.3.3 Alkylation of the Amide Nitrogen

Incorporation of a methyl group on an amide nitrogen is a commonly used strategy to increase peptide stability. N-Methylated analogs of endomorphin-2 show significantly improved stability while maintaining biological activity.[99] However, the conformation of the peptide backbone can be altered as well as its hydrogen bonding capability.[97]

### 2.4.3.4 Amide Bond Surrogate

This approach replaces an amide bond in the peptide backbone with a group that is not susceptible to hydrolysis. Examples include replacing the amide bond with

a reduced amide bond (-$CH_2NH$-) or with -$CH_2S$-. These replacements would eliminate cleavage at the site of modification, but also affect the flexibility, conformation and hydrophobicity of the molecule.[97]

### 2.4.3.5 Cyclization

Cyclization of a peptide can reduce the peptide's conformational flexibility which may reduce interaction with the active site of a peptidase.[97] Cyclization of [$Met^5$]enkephalin was been reported to improve blood-brain barrier permeability as well as pharmacological and pharmacokinetic activity.[100] A limitation for this approach is the feasibility of synthesizing the cyclic analog.[97]

### 2.4.3.6 Side-chain Modification

A simple approach is to replace the amino acids that are in the protease recognition site, with either natural or non-natural amino acids. Chlorination of the phenylalanine at the N-terminus of enkephalin prevents metabolism and improves blood-brain barrier permeability without loss of biological activity.[100, 101]

## *2.5 Conclusions*

There is significant potential for the use of KOR antagonists for the treatment of cocaine abuse. KOR antagonism has been shown to prevent relapse of cocaine seeking behavior in mice subjected to stress. Small molecule KOR antagonists have exceptionally long-lasting pharmacological activity - up to several weeks after one

dose. This long-lasting activity could be problematic for the therapeutic development of these drugs. Therefore, we are investigating peptides as KOR antagonists *in vivo*.

Peptides have been used as successful drugs. Although peptides often have shorter half-lives *in vivo* when compared to nonpeptide ligands, with appropriate modification they can have sufficiently long half-lives for use *in vivo*. The conditioned place preference tests showed promising results for arodyn in preventing stress-induced relapse of cocaine use. More development of peptide KOR antagonists may result in an effective treatment for stress-induced addiction relapse. Based on *in vivo* studies, arodyn is a promising lead for further development.

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# 3 Design, Synthesis and Metabolism of Arodyn Analogs

## 3.1 Introduction

Kappa opioid receptor (KOR) antagonists are increasingly gaining importance as potential new therapeutics for various disorders including drug addiction. KOR antagonists have shown antidepressant activity in the forced swim test in rats.[1] They have shown antianxiolytic activity in rats.[2] They can decrease the withdrawal signs in opioid addicted subjects (and therefore have potential application in the treatment of opioid addiction[3, 4]), and KOR antagonists have shown promise in the treatment of cocaine dependence by preventing stress induced relapse of drug seeking behavior.[5, 6]

Arodyn (Ac-Phe-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-$NH_2$), a dynorphin A (Dyn A) analog, is a potent and selective antagonist of KOR.[7] From the results in cocaine conditioned place preference assays in mice, arodyn shows potential in the treatment of stress-induced relapse of cocaine-seeking behavior.[5] Although arodyn has a relatively long duration of action *in vivo* (1-3 days following introcerebroventricular administration) it has a very short half-life in rat brain homogenate and in rat brain slices; arodyn is rapidly metabolized with complete disappearance in 15 minutes.[8] Analysis of the metabolism of arodyn in rat brain in our laboratory revealed several sites in the arodyn sequence that appear to be susceptible to proteolytic cleavage (Figure 3-1). A cleavage between proline and lysine was identified after the results of these metabolism studies.

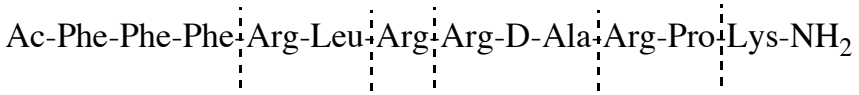


**Figure 3-1: Postulated cleavage sites for arodyn based on studies in striatal rat brain slice washings[8]**

Arodyn's cleavage sites show some similarities to those known for Dyn A. Thimet-oligopeptidase (EC 3.4.24.15) is a metallo-endopeptidase that has been reported to be involved in the metabolism of a variety of peptides at the Leu-Arg bond, including the dynorphins.[9] Endopeptidases selective for dibasic and monobasic sites in Dyn A have previously been reported.[10-14] Dynorphin A converting enzyme (EC 3.4.22) can cleave at $Arg^6$-$Arg^7$ and $Leu^5$-$Arg^6$ as well as at other monobasic sites, and $Ile^8$-$Arg^9$ is cleaved by Dyn A processing enzyme.[13] The metabolism of arodyn shows some similarities to Dyn A metabolism in that the cleavage sites are mono- or dibasic. Arodyn is cleaved at the N-terminus of every arginine – between residues 3-4 , 5-6, 6-7 and 8-9 peptide bonds in its sequence (a cleavage site at the N-terminus of lysine, at the 10-11 peptide bond was identified later).

Metabolically stable analogs of arodyn are of interest in order to develop systemically active peptides that could eventually lead to a therapeutically useful drug. Since cleavage of arodyn was observed at the N-termini of the arginines, these sites were targeted for modification. However, arodyn structure-activity relationship studies showed that the arginines are important residues for binding to KOR.[15] Thus, the modifications should retain the side chain functionality of arginine that is important for receptor binding. Backbone modifications, such as using N-methyl amino acids and replacing peptide bonds with reduced amide bonds, retain side chain

functionalities while hindering cleavage by peptidases at those residues.[16] With these considerations, we systematically replaced arginines in the arodyn sequence with N-methylarginine. We also incorporated reduced amide bonds between positions 3-4, 5-6, 6-7 and 8-9 in the sequence. Extending the peptide sequence by additional amino acids may also affect the metabolism rate – dynorphin metabolism has been found to vary by the length of the sequence, with longer sequences often being metabolized more slowly.[17] We extended the arodyn sequence with the addition of leucine in the 12$^{th}$ position and lysine in the 13$^{th}$ position to give [Leu$^{12}$]arodyn and [Leu$^{12}$,Lys$^{13}$]arodyn. These amino acids were chosen to mimic the Dyn A sequence. Here we describe the synthesis of these analogs and their metabolism in rat brain slices. Table 3-1 lists all of the analogs that were synthesized.

**Table 3-1: Synthesized arodyn analogs, listed by type of modification.**

| Arodyn Analogs | | | |
|---|---|---|---|
| NMeArg | Reduced Amide Bond | Extended | Other |
| [NMeArg$^{4}$] | [Phe$^{3}\Psi$(CH$_2$NH)Arg$^{4}$] | [Leu$^{12}$] | [Lys$^{6}$] |
| [NMeArg$^{6}$] | [Leu$^{5}\Psi$(CH$_2$NH)Arg$^{6}$] | [Leu$^{12}$,Lys$^{13}$] | |
| [NMeArg$^{7}$] | [Lys$^{6}\Psi$(CH$_2$NH)Arg$^{6}$] | | |
| [NMeArg$^{9}$] | [D-Ala$^{8}\Psi$(CH$_2$NH)Arg$^{9}$] | | |

## *3.2 Synthesis*

### 3.2.1 Extended Analogs and [Lys$^{6}$]arodyn

The extended analogs [Leu$^{12}$]arodyn and [Leu$^{12}$,Lys$^{13}$]arodyn as well as the analog [Lys$^{6}$]arodyn were synthesized using standard Fmoc (9-

fluorenylmethoxycarbonyl) solid phase synthesis on the peptide amide linker poly(ethylene glycol)-polystyrene (PAL-PEG PS) resin, as described in the experimental section. The crude peptides were purified by preparative HPLC, and the pure fractions were combined after analysis of the fractions by analytical HPLC. The purity of the combined fractions was confirmed by analytical HPLC in an acetonitrile/water (containing 0.1% TFA – trifluoroacetic acid) system and in a methanol/water (with 0.1% TFA) system (Table 3-2, the MS – mass sprectrometry – data is also listed).

### 3.2.2 N-Methylarginine Analogs

In order to best isolate the effects of individual modifications on the metabolism and fate of arodyn in rat brain slices, a single arginine was replaced with an N-methylarginine in each analog. All analogs were synthesized by Fmoc solid phase synthesis by a combination of automated and manual synthesis, using FmocNMeArg(Mtr) to incorporate NMeArg. Reaction times for coupling of the NMeArg and coupling the next amino acid in the sequence to NMeArg were somewhat longer than typical – 2-3 hours. Cleavage from the resin was done using modified Reagent K[18] (90% TFA, 5% thioanisole, 2.5% phenol and 2.5% 3,6-dioxa-1,8-octanedithiol)[19], in order to remove the Mtr (4-methoxy-2,3,6-trimethylbenzenesulfonyl) protecting group. The crude peptides were purified by preparative HPLC, and the pure fractions were combined after analysis of the fractions by analytical HPLC. The purity of the combined fractions was confirmed

by analytical HPLC in an acetonitrile/water (0.1% TFA) system and in a methanol/water (0.1% TFA) system (Table 3-2).

**Table 3-2: Analytical data for synthesized arodyn analogs.**

| Arodyn Analog | HPLC $R_z$(min)/purity(%) | | ESI-MS (*m/z*) | |
|---|---|---|---|---|
| | System 1[a] | System 2[b] | Calculated | Observed |
| [Leu$^{12}$] | 25.69/100 | 32.04/100[c] | $[M+3H]^{3+}$=550.0<br>$[M+4H]^{4+}$=412.8<br>$[M+2TFA+2]^{2+}$=938.5 | $[M+3H]^{3+}$=550.0<br>$[M+4H]^{4+}$=412.8<br>$[M+2TFA+2]^{2+}$=938.5 |
| [Leu$^{12}$Lys$^{13}$] | 24.01/98.3 | 33.64/100 | $[M+3H]^{3+}$=592.7<br>$[M+4H]^{4+}$=444.8 | $[M+3H]^{3+}$=593.0<br>$[M+4H]^{4+}$=444.8 |
| [NMeArg$^4$] | 24.88/100 | 40.79/100 | $[M+3H]^{3+}$=517.0<br>$[M+4H]^{4+}$= 388.0<br>$[M+2TFA+2]^{2+}$=888.5 | $[M+3H]^{3+}$=517.0<br>$[M+4H]^{4+}$= 388.0<br>$[M+2TFA+2]^{2+}$=889.0 |
| [NMeArg$^6$] | 28.10/99 | 41.67/98.5 | $[M+3H]^{3+}$=517.0<br>$[M+4H]^{4+}$= 388.0<br>$[M+2TFA+2]^{2+}$=888.5 | $[M+3H]^{3+}$=517.0<br>$[M+4H]^{4+}$=388.0<br>$[M+2TFA+2]^{2+}$=888.9 |
| [NMeArg$^7$] | 27.29/100 | 41.97/100 | $[M+2]^{2+}$=775.0<br>$[M+3H]^{3+}$=517.0<br>$[M+4H]^{4+}$= 388.0 | $[M+2]^{2+}$=775.0<br>$[M+3H]^{3+}$=517.0<br>$[M+4H]^{4+}$= 388.0 |
| [NMeArg$^9$] | 27.22/100 | 26.03/98.4[d] | $[M+3H]^{3+}$=517.0<br>$[M+4H]^{4+}$= 388.0<br>$[M+2TFA+2]^{2+}$=888.5 | $[M+3H]^{3+}$=517.0<br>$[M+4H]^{4+}$= 388.0<br>$[M+2TFA+2]^{2+}$=888.9 |
| [Phe$^3\Psi$(CH$_2$NH)Arg$^4$] | 23.74/99 | 37.69/99 | $[M+3H]^{3+}$=507.6<br>$[M+4H]^{4+}$= 381.0<br>$[M+2TFA+2]^{2+}$=875.0 | $[M+3H]^{3+}$=508.0<br>$[M+4H]^{4+}$= 381.2<br>$[M+2TFA+2]^{2+}$=875.0 |
| [Leu$^5\Psi$(CH$_2$NH)Arg$^6$] | 21.99/99 | 39.19/100 | $[M+3H]^{3+}$=507.6<br>$[M+4H]^{4+}$= 381.0<br>$[M+2TFA+2]^{2+}$=875.0 | $[M+3H]^{3+}$=507.6<br>$[M+4H]^{4+}$= 381.0<br>$[M+2TFA+2]^{2+}$=875.0 |
| [Lys$^6\Psi$(CH$_2$NH)Arg$^7$] | 22.62/99 | 37.14/100 | $[M+3H]^{3+}$=498.3<br>$[M+TFA+2]^{2+}$=804.0 | $[M+3H]^{3+}$=498.3<br>$[M+TFA+2]^{2+}$=804.0 |
| [D-Ala$^8\Psi$(CH$_2$NH)Arg$^9$] | 21.97/99 | 39.81/100 | $[M+3H]^{3+}$=507.6<br>$[M+4H]^{4+}$= 381.0<br>$[M+2TFA+2]^{2+}$=875.0 | $[M+3H]^{3+}$=507.6<br>$[M+4H]^{4+}$= 381.0<br>$[M+2TFA+2]^{2+}$=875.0 |
| [Lys$^6$] | 23.23/100 | 41.69/100 | $[M+3H]^{3+}$=503.0 | $[M+3H]^{3+}$=503.0 |

[a] System 1: Solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in acetonitrile.
[b]System 2: Solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in methanol. The gradient for both systems was 5-50% solvent B, except where noted, over 45 min and the flow rate was 1mL/min.
[c] 20% to 60% solvent B over 45 min.
[d] 20% to 70% solvent B over 50 min.

### 3.2.3 Amides and Aldehydes

The reduced amide bond analogs were synthesized using reductive amination with amino aldehydes. These aldehydes were prepared from the corresponding Weinreb amides.

The Weinreb amides were synthesized starting from Fmoc-protected amino acids as described in the experimental section and shown in Figure 3-2. The amino acids used were: Fmoc-Phe-OH, Fmoc-D-Ala-OH, Fmoc-Leu-OH and Fmoc-Lys(Boc)-OH. Analytical HPLC and MS were used to confirm purity and identity of the Weinreb amides. The mass of the impurities in MS matched the mass of the expected by-products of PyBOP. The amides were then converted to aldehydes without purification.

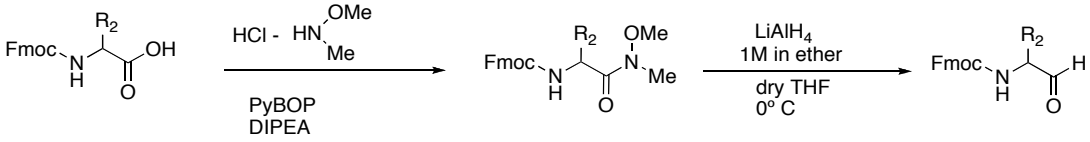


**Figure 3-2: Weinreb amide synthesis, followed by reduction to an aldehyde**

Fmoc-proteceted amino aldehydes were synthesized from the Weinreb amides (Figure 3-2) as described in the experimental section. The amino aldehydes Fmoc-Phe-H, Fmoc-D-Ala-H, Fmoc-Leu-H and Fmoc-Lys(Boc)-H were successfully obtained. Proton NMR was used to confirm the presence of the aldehyde proton before proceeding with the reductive amination. The aldehydes were used within 18 hours of preparation, without purification.

### 3.2.4 Reduced Amide Bond Analogs

The reduced amide bond analogs were synthesized using the appropriate aldehydes. Lysine was substituted for arginine in position 6 in the analog that would have required an arginine aldehyde because Fmoc-protected lysine aldehydes were simpler to synthesize than arginine aldehydes. However, the lysine analog may show some differences in activity despite the similarity in charges. The cleavage of arodyn appears to occur on the N-terminal side of the arginine residues, and substitution of arginine by a lysine could alter the cleavage at the N-terminus of the lysine residue in addition to at the reduced amide bond. Therefore, [$Lys^6$]arodyn was also synthesized for comparison in the metabolism studies of the effect of only the lysine substitution. [N-Me-$Arg^7$]arodyn can also be used for comparison, as this analog has the same peptide bond modified as the $Lys^6$ reduced amide analog but maintains arginine at position 6.

The reductive amination of the deprotected peptide with the aldehyde and $NaCNBH_3$ was performed for 3-5 hours. The reactions were followed by the ninhydrin test, which gave an orange/yellow color upon completion of the reaction. Aliquots taken at this step indicated no double alkylation occurred (confirmed by mass specrometry). The remaining amino acids in the peptide sequence were then introduced using standard procedures with PyBOP and HOBt as the coupling reagents.

Acetylation of the completed peptide was first attempted using acetic anhydride. Mass spectra of the products revealed double acetylation, indicating that

the reduced amide bond was acetylated in addition to the amine terminus. Mass spectra of this, or earlier steps in the sequence, did not indicate any coupling of amino acids to the reduced amide bond site. A milder method of acetylation with acetylimidazole and DIEA (*N,N*-diisopropylethylamine) produced no double acetylation even under long reaction times (2-3 h). The crude peptides were purified by preparative HPLC, and the pure fractions were combined after analysis of the fractions by analytical HPLC. The purity of the combined fractions was confirmed by analytical HPLC in an acetonitrile/water (0.1% TFA) system and in a methanol/water (0.1% TFA) system (Table 3-2).

## *3.3 Binding Studies*

Preliminary binding studies are being carried out in Dr. Thomas Murray's laboratory at Creighton University using [$^3$H]diprenorphine as the radioligand for KOR. The initial results of these studies are summarized in Table 3-3. The extended analogs and N-methylarginine analogs showed roughly 4- to 5-fold lower KOR affinity than arodyn ($K_i$ = 10 nM[7]). Studies on the binding of the reduced amide bond analogs are underway. All of these peptides will also be evaluated for affinity at the μ and δ opioid receptors to determine selectivity for KOR.

**Table 3-3: Binding to KOR of arodyn analogs.**

| Arodyn Analog | $K_i$ ± SEM (nM) |
|---|---|
| [Leu$^{12}$] | 52.7 ± 3.6 |
| [Leu$^{12}$Lys$^{13}$] | 36.1 ± 2.7 |
| [NMeArg$^4$] | 50.1 ± 4.0 |
| [NMeArg$^6$] | 67.5 ± 4.7 |
| [NMeArg$^7$] | 66.1 ± 4.3 |
| [NMeArg$^9$] | 78.5 ± 5.9 |

## 3.4 Metabolism Studies

Initially, five peptides were chosen for the metabolism studies. Both the reduced amide bond and N-methylarginine substitutions were expected to inhibit proteolytic cleavage at the site of modification.[16] The reduced amide bond series of analogs was investigated in the initial studies. In addition to the reduced amide bond series an extended analog, [Leu$^{12}$,Lys$^{13}$]arodyn, was also chosen for study.

Striatal brain slices were used for our metabolism experiments. The slice washings are expected to be composed primarily of the extracellular enzyme fraction as opposed to rat brain homogenate, which is likely to contain both intra- and extracellular proteases. Arodyn is expected to be metabolized by extracellular enzymes *in vivo*; thus the rat brain slice experiments are expected to be more consistent with *in vivo* metabolism of these peptides. Reed *et al.* observed similar cleavage products for β-endorphin *in vivo* and in washes of striatal slices.[20] Extracellular enzymes have been implicated in the metabolism of Dyn A in a number of studies,[14, 21] including *in vivo* experiments in rat striata.[13]

The peptides were incubated at 37 °C with washings from striatal rat brain slices, with and without an aminopeptidase inhibitor (see experimental section for details).[20] Inclusion of the aminopeptidase inhibitor bestatin enabled detection of the larger fragments of ardoyn analogs that, in the absence of the inhibitor, could undergo further degradation. For example, cleavage between the Phe$^3$-Arg$^4$ residues of arodyn would leave an unprotected arginine at the N-terminus of one fragment, which could then be further cleaved by aminopeptidases. The presence of a carboxypeptidase

inhibitor in the experiment was found to have no effect on the types of metabolites found in earlier metabolism studies on the parent peptide arodyn.[8] Aliquots, taken at 3, 6, 10, 15, 30 and 60 minutes, were immediately placed on ice to stop further metabolism. Each aliquot was mixed with the matrix CHCA (α-cyano-4-hydroxycinnamic acid) and then MALDI-MS (matrix-assisted laser desorption ionization mass spectrometry) was used to identify the metabolites. This method gives qualitative insight into the degradation of the peptides. Table 3-4 summarizes the major metabolites observed for each analog. The results are reported for the studies that were carried out in the presence of aminopeptidase inhibitor (the results with no inhibitor were similar and are given in Appendix 1). Small metabolites (*m/z* < 700) are difficult to detect with this method due to background peaks (from the rat brain slice washings and the matrix) which show up in this region. In addition, more highly charged fragments are more easily detected – for arodyn and analogs the C-terminal fragments are often detected while the N-terminal fragments are not. Figures showing the cleavages sites for each analog (and $(M+H)^+$ values) are presented later in this chapter.

The study of the metabolism of these peptides using rat brain slices using MALDI MS proved to be useful in identifying several metabolites of arodyn.[8] Even though variability in the intensity was observed between time points for each analog, most of the analogs showed a consistent cleavage pattern predominantly involving the basic residues in the peptide sequences. The modifications at the arginine residues

restricted cleavage at these residues. Most peptides showed rapid proteolysis within the first 10 min of incubation with washings from striatal rat brain slices.

Spectra peaks were considered to be significant with a signal to noise ratio of greater 25:1, with the exception of the blank where peaks were considered to be significant at a signal to noise ratio of greater than 10:1. Appendix 2 contains the spectra for the blank, [$Leu^{12}$,$Lys^{13}$]arodyn at 3, 6, 10, 15, 30 and 60 minutes as well as at 15 minutes incubation for [$Phe^{3}\Psi(CH_2NH)Arg^{4}$]arodyn, [$Leu^{5}\Psi(CH_2NH)Arg^{6}$], [$Lys^{6}\Psi(CH_2NH)Arg^{7}$], [D-$Ala^{8}\Psi(CH_2NH)Arg^{9}$], [$NMeArg^{6}$] and [$NMeArg^{7}$]arodyn.

**Table 3-4: Metabolites of arodyn analogs observed following incubation with washings from rat brain slices in the presence of bestatin.[a]**

| Arodyn analog / (mol. weight) | Metabolites (*m/z* observed) | | | | | |
|---|---|---|---|---|---|---|
| | 3min | 6min | 10min | 15min | 30min | 60min |
| [Leu$^{12}$,Lys$^{13}$] / (1776) | 1777, 1293, 1024 | 1777, 1293, 1165, 1052, 1024, 924, 867 | 1777, 1293, 1052, 1024, 924, 867 | 1777, 1293, 1052, 1024, 924, 867 | 1777[c], 867 [c] | No peaks[b] |
| [Phe$^{3}\Psi$($CH_2NH$) Arg$^{4}$] / (1520) | 1522, 1394, 914, 757 | 1522, 1394, 914, 757 | 1522, 1394, 914, 757 | 1522, 1394, 914, 757 | 1522, 1394, 914, 757 | 914, 757 |
| [Leu$^{5}\Psi$($CH_2NH$) Arg$^{6}$] / (1520) | 1522 | 1522, 1394 | 1522, 1394 | 1522, 1394, 1141, 1038 | 1522, 1394, 1141, 1038 | 1522, 1394, 1141, 1038, 911 |
| [Lys$^{6}\Psi$($CH_2NH$) Arg$^{7}$] / (1491) | 1493, 1367, 884 | 1493, 1367, 884 | 1493, 1367, 1010, 884 | 1493, 1367, 1010, 884 | 1493, 1367, 1010, 884 | 1367, 1010, 884 |
| [D-Ala$^{8}\Psi$($CH_2NH$) Arg$^{9}$] / (1520) | 1522, 1038 | 1522, 1038 | 1522, 1038 | 1522, 1038 | No peaks[b] | No peaks[b] |
| [NMeArg$^{6}$] / (1547) | 1548, 1066 | 1548, 1066, 939 | 1548, 1066, 939 | 1548[d], 1066, 939 | 1066, 939 | 939 |
| NMeArg$^{7}$] / (1547) | 1548, 1066 | 1548, 1066, 939 | 1548, 1066, 939 | 1548, 1066, 939 | 1066, 939 | 939 |
| Arodyn | 1535, 441 (0 min) | 1535, 1052, 782, 626, 441 (5 min) | ND | 782, 636, 441 | 782, 626, 441 | ND |
| Blank | 1450, 1411, 1376, 772, 757 | ND | ND | ND | ND | ND |

[a]Internal standard peaks: 1466, 1488 and 1504.
[b]Internal standard was visible but no metabolite peaks were observed above the background.
[c]Peak intensities very low (possibly difficult to read MALDI spot), these peaks were clear in non-bestatin inhibited experiment at 30 min.
[d] Signal/noise ratio is 17.57
ND = not determined

[Leu$^{12}$,Lys$^{13}$]arodyn was degraded into several metabolites. The parent peptide was still detectable up to 30 minutes (in contrast to arodyn which was not detected after 5 minutes). The cleavage sites are shown in Figure 3-3.

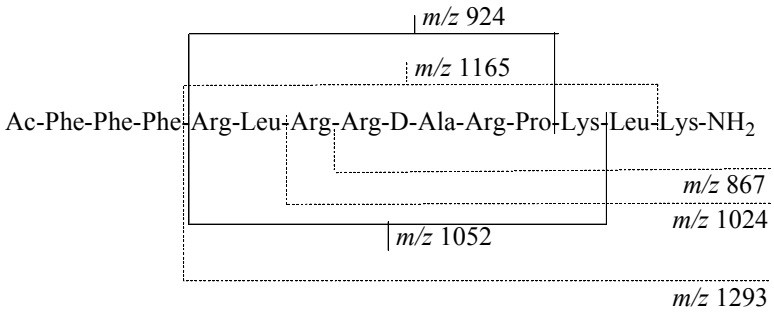


**Figure 3-3: Observed fragments for the extended analog [Leu$^{12}$Lys$^{13}$]arodyn.**

At 3 minutes cleavages between Phe$^3$-Arg$^4$ (*m/z* 1293) and Leu$^5$-Arg$^6$ *(m/z* 1024) were detected. The 1293 fragment appeared to be cleaved further to yield smaller metabolites (*m/z* 1165 and 1052) at 6 min. Additional cleavages at this time point were between Arg$^6$-Arg$^7$ (*m/z* 867) and Pro$^{10}$-Lys$^{11}$ (*m/z* 1165). Similar metabolites are seen at 10, 15 and 30 min. The parent peptide is still detectable after 30 min. The observed fragments are listed in Table 3-5.

**Table 3-5: Relative intensity (% of internal standard) of detected metabolites of [Leu$^{12}$,Lys$^{13}$]arodyn.**

| Fragment Mass (*m/z*) | Sequence | Time (min) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 3 | 6 | 10 | 15 | 30 | 60 |
| 1777 | AcPhe-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-Leu-Lys-$NH_2$ | 22 | 16 | 8 | 6 | * | |
| 1293 | Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-Leu-Lys-$NH_2$ | 12 | 39 | 19 | 9 | | |
| 1165 | Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-Leu | | 5 | | | | |
| 1052 | Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys | | 14 | 12 | 13 | | |
| 1024 | Arg-Arg-D-Ala-Arg-Pro-Lys-Leu-Lys-$NH_2$ | 5 | 20 | 27 | 28 | | |
| 924 | Arg-Leu-Arg-Arg-D-Ala-Arg-Pro | | 9 | 8 | 9 | | |
| 867 | Arg-D-Ala-Arg-Pro-Lys-Leu-Lys-$NH_2$ | | 13 | 24 | 22 | * | |

*Peak intensities too low to read.

There were no detectable metabolites or parent peptide seen after 30 minutes. The internal standard peak was still clear at 60 minutes, but no metabolites were seen

above the background. The only cleavage observed that did not occur at the N-terminus of an arginine or lysine was between Lys[11]-Leu[12]. However, this may be a secondary cleavage - the Leu[12] may have been cleaved by a carboxypeptidase following cleavage of Leu[12]-Lys[13].Thus, the extended analog [Leu[12],Lys[13]]arodyn was cleaved at the N-termini of lysine and arginine residues and had greater stability than arodyn.

For [Phe[3]Ψ($CH_2$NH)Arg[4]]arodyn, the parent compound was detectable until 30 minutes. Only three metabolites were detected, corresponding to cleavages between Leu[5]-Arg[6] (*m/z* 757), Arg[6]-Arg[7] (*m/z* 914) and Pro[10]-Lys[11] (*m/z* 1394), all of which were detected as early as 3 minutes. Figure 3-4 shows the observed fragments and Table 3-6 lists these relative intensities of these fragments by the times at which they were detected.

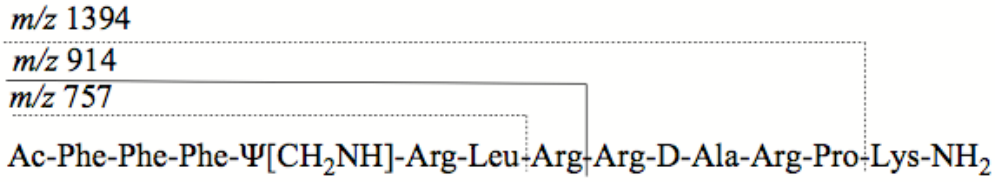


**Figure 3-4: Observed fragments for [Phe[3]Ψ($CH_2$NH)Arg[4]]arodyn.**

**Table 3-6: : Relative intensity (% of internal standard) of detected metabolites of [Phe[3]Ψ($CH_2$NH)Arg[4]]arodyn.**

| Fragment Mass (*m/z*) | Sequence | Time (min) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 3 | 6 | 10 | 15 | 30 | 60 |
| 1522 | Ac-Phe-Phe-Phe–Ψ[$CH^2$NH]-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-$NH_2$ | 37 | 45 | 43 | 34 | 10 | |
| 1394 | Ac-Phe-Phe-Phe–Ψ[$CH^2$NH]-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro | 15 | 15 | 17 | 17 | 10 | |
| 914 | Ac-Phe-Phe-Phe–Ψ[$CH^2$NH]-Arg-Leu-Arg | 14 | 12 | 17 | 20 | 21 | 35 |
| 757 | Ac-Phe-Phe-Phe–Ψ[$CH^2$NH]-Arg-Leu | 19 | 18 | 26 | 35 | 26 | 24 |

For [Leu$^5$Ψ(CH$_2$NH)Arg$^6$]arodyn, parent compound was detected at 60 min - longer than any other analog and much longer than arodyn. This compound has four cleavage sites, each at the N-termini of arginine and lysine. The observed fragments are shown in Figure 3-5. The cleavage at Pro$^{10}$-Lys$^{11}$ was detected at 6 minutes and other cleavages (Arg$^6$-Arg$^7$ and D-Ala$^8$-Arg$^9$) were not detected until 15 minutes.

**Table 3-7: Relative intensity (% of the internal standard) of detected metabolites of [Leu$^5$Ψ(CH$_2$NH)Arg$^6$]arodyn.**

| Fragment Mass (*m/z*) | Sequence | Time (min) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 3 | 6 | 10 | 15 | 30 | 60 |
| 1522 | Ac-Phe-Phe-Phe-Arg-Leu- Ψ[CH$^2$NH]-Arg-Arg-D-Ala-Arg-Pro-Lys-NH$_2$ | 59 | 59 | 33 | 41 | 21 | 7 |
| 1394 | Ac-Phe-Phe-Phe-Arg-Leu- Ψ[CH$^2$NH]-Arg-Arg-D-Ala-Arg-Pro | | 18 | 10 | 30 | 29 | 19 |
| 1141 | Ac-Phe-Phe-Phe-Arg-Leu- Ψ[CH$^2$NH]-Arg-Arg-D-Ala | | | | 9 | 12 | 10 |
| 1038 | Arg-Leu- Ψ[CH$^2$NH]-Arg-Arg-D-Ala-Arg-Pro-Lys-NH$_2$ | | | | 12 | 13 | 4 |
| 911 | Ac-Phe-Phe-Phe-Arg-Leu- Ψ[CH$^2$NH]-Arg | | | | | | 7 |

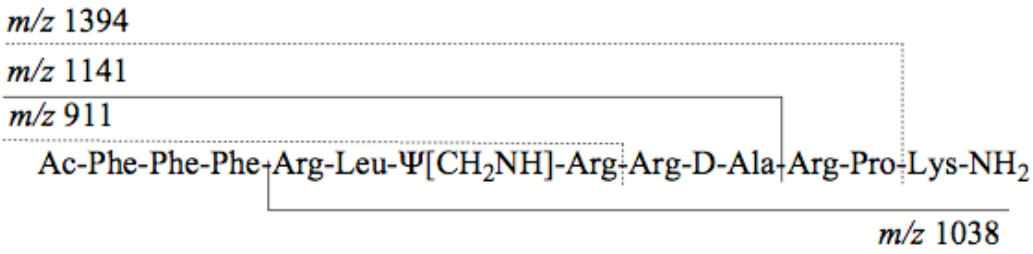


**Figure 3-5: Observed fragments for [Leu$^5$Ψ(CH$_2$NH)Arg$^6$]arodyn.**

Metabolism again occurred mainly at the N-termini of argnine and lysine residues for [Lys$^6$Ψ(CH$_2$NH)Arg$^7$]arodyn, (see Figure 3-6). The parent compound

was seen after 30 minutes of incubation. There was no cleavage detected at the Leu$^5$-Lys$^6$ bond. Cleavages were seen as early as 3 minutes at Phe$^3$-Arg$^4$ and Pro$^{10}$-Lys$^{11}$ (*m/z* 884, corresponding to cleavage of both the 3-4 and 10-11 bonds and *m/z* 1367). Cleavage of just the 3-4 bond (*m/z* 1010) was also detected at 10 minutes and later. Table 3-8 summarizes the fragments found and their relative intensities.

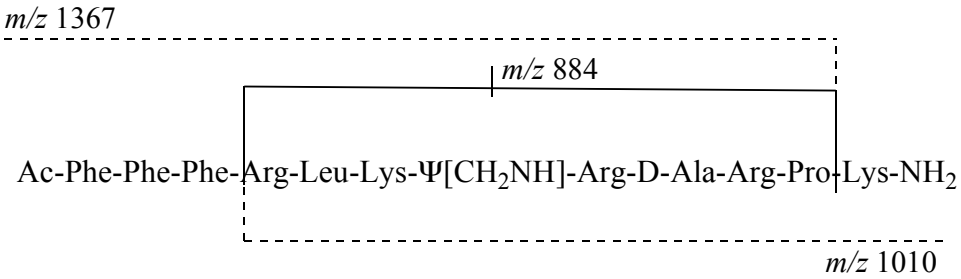


**Figure 3-6: Observed fragments for [Lys$^6$Ψ(CH$_2$NH)Arg$^7$]arodyn.**

**Table 3-8: Relative intensity (% of the internal standard) of detected metabolites of [Lys$^6$Ψ(CH$_2$NH)Arg$^7$]arodyn.**

| Fragment Mass (*m/z*) | Sequence | Time (min) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 3 | 6 | 10 | 15 | 30 | 60 |
| 1493 | Ac-Phe-Phe-Phe-Arg-Leu-Lys-Ψ[CH$^2$NH]-Arg-D-Ala-Arg-Pro-Lys-NH$_2$ | 57 | 54 | 29 | 32 | 11 | |
| 1367 | Ac-Phe-Phe-Phe-Arg-Leu-Lys-Ψ[CH$^2$NH]-Arg-D-Ala-Arg-Pro | 4 | 8 | 10 | 17 | 11 | 11 |
| 1010 | Arg-Leu-Lys-Ψ[CH$^2$NH]-Arg-D-Ala-Arg-Pro-Lys-NH$_2$ | | | 12 | 41 | 12 | 14 |
| 884 | Arg-Leu-Lys-Ψ[CH$^2$NH]-Arg-D-Ala-Arg-Pro | 7 | 9 | 13 | 12 | 4 | 4 |

Only one metabolite was seen for [D-Ala$^8$Ψ(CH$_2$NH)Arg$^9$]arodyn (Figure 3-7 and Table 3-9), yet the parent compound only survived until 15 minutes. Although the internal standard was clearly visible at 30 and 60 minutes, no metabolites of the

arodyn analog could be seen above the background. This could be due to further metabolism resulting in small metabolites that were not detectable using the MALDI.

**Table 3-9: Relative intensity (% of the internal standard) of detected metabolites of [D-Ala$^8$Ψ(CH$_2$NH)Arg$^9$]arodyn.**

| Fragment Mass (*m/z*) | Sequence | Time (min) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 3 | 6 | 10 | 15 | 30 | 60 |
| 1522 | Ac-Phe-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Ψ[CH$^2$NH]-Arg-Pro-Lys-NH$_2$ | 30 | 11 | 9 | 4 | | |
| 1038 | Arg-Leu-Arg-Arg-D-Ala- Ψ[CH$^2$NH]-Arg-Pro-Lys-NH$_2$ | 8 | 6 | 5 | 4 | | |


**Figure 3-7: Observed fragments for [D-Ala$^8$Ψ(CH$_2$NH)Arg$^9$]arodyn.**

The initial metabolism studies showed that the most stable peptide was [Leu$^5$Ψ(CH$_2$NH)Arg$^6$]arodyn, which has the 5-6 bond stabilized as the reduced amide bond, where the parent peptide was detected after 60 minutes. The 5-6, i.e. Leu-Arg, cleavage is common among dynorphin derivatives and could be due to the enzyme thimet-oligopeptidase.[9] This bond can also be cleaved by dynorphin converting enzyme.[14]

[Lys$^6$Ψ(CH$_2$NH)Arg$^7$]arodyn did not show the 5-6 bond cleavage. This could have been due to the substitution of lysine for arginine at residue 6. In order to investigate this, the metabolism of [NMeArg$^7$]arodyn was studied. This peptide also has a stabilized 6-7 bond, like [Lys$^6$Ψ(CH$_2$NH)Arg$^7$]arodyn, but maintains an

arginine at the 6th position instead of a lysine. A 5-6 cleavage in this analog would suggest that the presence of arginine could be important for the metabolism at this site and that replacement of arginine at this position by the basic amino acid lysine is deleterious to proteolysis of this bond (indicating enzyme selectivity for arginine over lysine). In addition, [NMeArg$^6$]arodyn was chosen to investigate whether stabilization of the 5-6 bond with an N-methyl group would result in a peptide as stable as the analog [Leu$^5\Psi(CH_2NH)$]Arg$^6$arodyn.

As shown in Table 3-4 [NMeArg$^6$]arodyn was not detected after 15 minutes, unlike [Leu$^5\Psi(CH_2NH)$Arg$^6$]arodyn, which was observed for 60 minutes. Two major metabolites were seen for [NMeArg$^6$]arodyn, one corresponding to a cleavage between Phe$^3$-Arg$^4$ (*m/z* 1066) and another between both Phe$^3$-Arg$^4$ and Pro$^{10}$-Lys$^{11}$ (*m/z* 939). This is shown in Figure 3-8. The spectrum obtained following 15 minutes incubation is shown in Figure xxii in Appendix 2. Thus, the N-methylarginine analog had fewer metabolites (no cleavage of bonds 6-7 or 8-9) but faster metabolism of the parent peptide than the reduced amide bond analog.

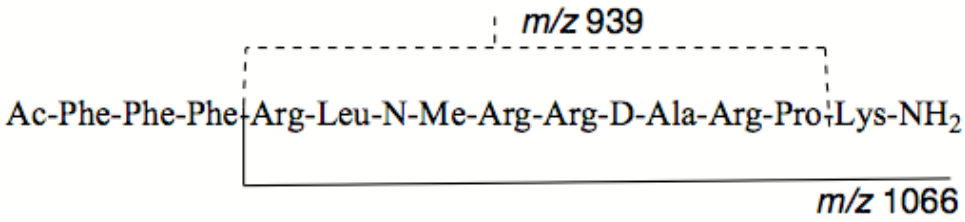


**Figure 3-8: Observed fragments of [NMeArg$^6$]arodyn.**

**Table 3-10: Relative intensity (% of the internal standard) of detected metabolites of [NMeArg$^6$]arodyn.**

| Fragment Mass (*m/z*) | Sequence | Time (min) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 3 | 6 | 10 | 15 | 30 | 60 |
| 1548 | Ac-Phe-Phe-Phe-Arg-Leu-NMeArg-Arg-D-Ala-Arg-Pro-Lys-$NH_2$ | 25 | 40 | 26 | 50 | | |
| 1066 | Arg-Leu-NMeArg-Arg-D-Ala-Arg-Pro-Lys-$NH_2$ | 30 | 144* | 185* | 366* | 55 | 100 |
| 939 | Arg-Leu-NMeArg-Arg-D-Ala-Arg-Pro | | 64 | 112* | 638* | 184* | 45 |

*Metabolite peak is larger than internal standard.

[NMeArg$^7$]arodyn had the same 6-7 bond stabilized as [Lys$^6\Psi$Arg$^7$]arodyn, but the parent [NMeArg$^7$]arodyn was not observed after 15 minutes. The spectrum for the 15 minute incubation is shown in Figure xxiii in Appendix 2. The cleavages for the latter peptide were similar to those for [N-Me-Arg$^6$]arodyn, as can be seen in Figure 3-9. No cleavage was seen at the 5-6 bond for [NMeArg$^7$]arodyn. This would suggest that the lysine replacement in [Lys$^6\Psi$($CH_2$NH)Arg$^7$]arodyn did not affect the metabolism at this site.

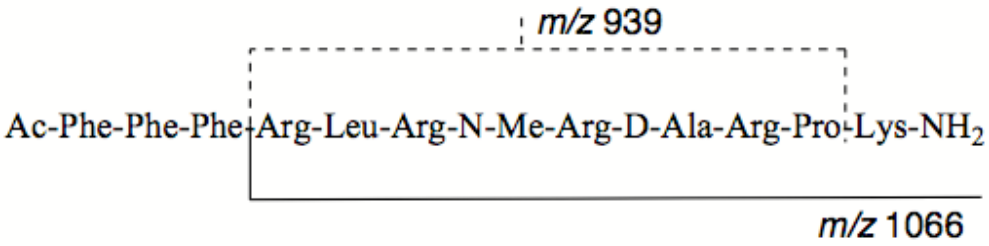


**Figure 3-9: Observed fragments of [NMeArg$^7$]Arodyn.**

**Table 3-11: Relative intensities (to the internal standard) of detected metabolites of [NMeArg$^7$]arodyn.**

| Fragment Mass (*m/z*) | Sequence | Time (min) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 3 | 6 | 10 | 15 | 30 | 60 |
| 1548 | Ac-Phe-Phe-Phe-Arg-Leu-Arg-NMeArg-D-Ala-Arg-Pro-Lys-$NH_2$ | 24 | 13 | 46 | 25 | | |
| 1066 | Arg-Leu-Arg-NMeArg-D-Ala-Arg-Pro-Lys-$NH_2$ | 13 | 15 | 26 | 16 | 9 | |
| 939 | Arg-Leu-Arg-NMeArg-D-Ala-Arg-Pro | | 10 | 26 | 26 | 80 | 169* |

*Internal standard not largest peak.

## *3.5 Summary*

Eleven new arodyn analogs were synthesized to find more metabolically stable peptides. Extended analogs and backbone modifications were the focus of these syntheses. A series of N-methylarginine analogs was prepared with single N-methyl-arginine replacements for each arginine residue. A series of reduced amide bond analogs was also synthesized, each one containing a reduced amide bond at a known site of cleavage (at the N-terminus of each arginine residue). Also, two extended arodyn analogs were synthesized in order to investigate the effects of peptide length on proteolysis.

One extended analog, the reduced amide bond series and two N-methylarginine analogs were chosen for the metabolism studies. Cleavages for all of the analogs were generally found at the N-termini of basic residues (arginine and lysine), consistent with the previous results for arodyn. These results also have similarities to cleavage patterns for dynorphin[13] in that cleavages are at mono- and dibasic sites. However, the enzymes responsible for cleaving these arodyn analogs

seem to show a selectivity for the N-terminus of basic residues, a selectivity which is not seen in dynorphin A metabolism.

The $Pro^{10}$-$Lys^{11}$ cleavage was seen for every analog except [D-$Ala^{8}\Psi(CH_2NH)Arg^{9}$]arodyn. Both the N-methylarginine and reduced amide bond analogs showed extensive cleavage at the $Pro^{10}$-$Lys^{11}$ bond. This cleavage appears at the earliest time point (3 minutes) in 5 out of the 7 analogs. Amidation of the C-terminus and a nearby proline residue were not sufficient to prevent cleavage at this site.

The $Phe^{3}$-$Arg^{4}$ bond was cleaved in all but one analog – the analog with stabilization at that site ([$Phe^{3}\Psi(CH_2NH)Arg^{4}$]arodyn). This cleavage was seen at 3 minutes for 5 out of 7 analogs. The parent [$Phe^{3}\Psi(CH_2NH)Arg^{4}$]arodyn was detected until 30 minutes, longer than arodyn (which was not detectable by 15 minutes) but shorter than the longest lasting analog ([$Leu^{5}\Psi(CH_2NH)Arg^{6}$]arodyn) tested in these studies.

Other common cleavage sites included $Arg^{6}$-$Arg^{7}$, the cleavage products of which were observed for three of the analogs ([$Leu^{12}$,$Lys^{13}$]-, [$Phe^{3}\Psi(CH_2NH)Arg^{4}$]- and [$Leu^{5}\Psi(CH_2NH)Arg^{6}$]). For the reduced amide analog with 6-7 bond stabilization ([$Lys^{6}\Psi(CH_2NH)Arg^{7}$]arodyn), the parent compound was detectable until 30 minutes. $Leu^{5}$-$Arg^{6}$ was cleaved in only two of the analogs ([$Phe^{3}\Psi(CH_2NH)Arg^{4}$]arodyn and ([$Leu^{12}$,$Lys^{13}$]arodyn). The analog with the 5-6 reduced amide bond, [$Leu^{5}\Psi(CH_2NH)Arg^{6}$]arodyn, showed the greatest stability, with the parent compound detectable at 60 minutes. In earlier research on reduced

amide bond analogs of Dyn A, it was found that stabilization of the 5-6 bond with a reduced amide bond (to give the analog [Leu$^5\Psi$($CH_2$NH)Arg$^6$]Dyn A(1-11)$NH_2$,) produced an analog that had a half-life of >500 minutes in mouse brain homogenate.[22] They also found this 5-6 bond was one of the most frequently cleaved bonds in Dyn A.[22] Cleavage of D-Ala$^8$-Arg$^9$ was only seen for the analog [Leu$^5\Psi$($CH_2$NH)Arg$^6$]arodyn.

Given the SAR of dynorphin A and the "message-address" concept,[23] cleavages in the "message" region of the peptide (residues 1-4) would produce metabolites lacking activity at opioid receptors. Cleavages in the rest of the peptide (the "address" region) may produce fragments with decreased KOR selectivity but that maintain activity at opioid receptors. Most of the cleavages for the analogs studied here occur in the address region, with one cleavage (of the 3-4 bond) that occurs late in the message sequence.

Replacement of the 3-4, 5-6 or 6-7 peptide bonds with a reduced amide bond produced the most stable analogs – these compounds survived up to 30 to 60 minutes. However, corresponding N-methylarginine substitutions did not produce equally stable compounds. Neither [NMeArg$^6$]arodyn nor [NMeArg$^7$]arodyn survived beyond 15 minutes in the metabolism studies. One reason for this unexpected observation could be due to the reduced amide bond imparting an additional positive charge to the peptide backbone, which may alter peptidase binding. The reduced amide bond could also alter the conformations of the peptides, which would change their binding to the peptidases. In either case, the reduced amide bond analogs could also have altered

receptor binding. Binding studies are in progress for these analogs to determine if KOR binding has been affected.

## *3.6 Experimental*

### 3.6.1 Materials

Standard Fmoc-protected amino acids were purchased from Bachem (King of Prussia, PA), Calbiochem-Novabiochem (San Diego, CA), Applied Biosystems (Foster City, CA), or Peptides International (Louisville, KY). The unnatural amino acid Fmoc-N-Me-Arg(Mtr)-OH was purchased from ChemImpex International (Wood Dale, IL). Fmoc-PAL-PEG-PS resin, DIEA and 7-azabenzotriazol-1-yloxy-tris-(pyrrolidino)phosphonium hexafluorophosphate (PyAOP) were purchased from Applied Biosystems. 1-Hydroxy-7-azabenzotriazole (HOAt) was purchased from GenScript (Piscataway, NJ). 1-Hydroxybenzotriazole (HOBt) was purchased from Fisher Scientific. Benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) was purchased from Novabiochem. Lithium aluminum hydride, *N,O*-dimethylhydroxylamine hydrochloride, the MALDI matrix α-cyano-4-hydroxycinnamic acid (CHCA) and other reagents were purchased from Sigma-Aldrich (St. Lous, MO). All HPLC-grade solvents (AcOH, MeCN, diethyl ether, *N,N*-dimethylformamide (DMF), dichloromethane (DCM), and MeOH) used for peptide synthesis or HPLC analysis were obtained from Fisher Scientific. TFA for HPLC analysis was also purchased from Fisher Scientific.

### 3.6.2 Synthesis of Weinreb Amides

Synthesis of amides and aldehydes followed a procedure adapted from Meyer *et al*.[24] Fmoc-Phe-OH (194 mg, 0.5 mmol) and PyBOP (286 mg, 0.55 mmol, 1.15 equiv) were dissolved in DCM (5 mL). DIEA (0.3 mL, 1.5 mmol, 3 eqiuv) was added and the solution was stirred for several minutes. *N,O*-Dimethylhydroxylamine hydrochloride (59 mg, 0.6 mmol, 1.2 equiv) dissolved in DCM (5 mL) was added and the reaction was stirred for 1 h. The progress of the reaction was followed with TLC (1:1 EtOAc/hexane). DCM (10 mL) was added and the solution was washed three times with each of the following: 3N HCl, water, saturated $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$, filtered and then concentrated. A colorless oil was obtained. MS showed impurities that were possible PyBOP byproducts. The product was used without further purification. ESI MS: 431.2 ($M+H^+$), byproducts: 258.2, 515.3, 537.3. The same byproduct peaks were present in all four different amides synthesized. The MS results are consistent with known syntheses.[25]

Other Weinreb amides were synthesized using the same procedure as above and the MS results are consistent with known syntheses: Fmoc-D-Ala-NOMe(Me) (MS 377.1, $M+Na^+$)[25], Fmoc-Leu-NOMe(Me) (397.2 $M+H^+$)[25] and Fmoc-Lys(Boc)-NOMe(Me) (512.7 $M+H^+$).[26]

### 3.6.3 Synthesis of Aldehydes

Fmoc-protected phenylalanine Weinreb amide (1590 mg, ~0.35 mmol) was dissolved in dry THF (4 mL) and the solution was cooled to 0 $^{\circ}C$ under $N_2$. $LiAlH_4$

(1M solution in ether, 0.7 mL) was added dropwise and the progress of the reaction was followed by TLC (1:1 EtOAc:hexane). After 2 h, 1M $KHSO_4$ solution (20 mL) and EtOAc (10 mL) was added at 0 °C to quench the remaining $LiAlH_4$. The reaction was stirred until it went clear, and then the layers were separated. The organic layer was washed twice with 1M $KHSO_4$ and once with brine, then dried over $Na_2SO_4$, filtered and concentrated to give a yellow oil (91 mg, ~0.24 mmol, ~70%). Proton NMR was used to confirm presence of the aldehyde ($CDCl_3$ δ 9.65 (C**H**O)). This was consistant with known reports.[27] The aldehyde was frozen and used within 18 h without further purification.

Fmoc-D-Ala-H ($CDCl_3$ δ 9.57 (C**H**O))[28], Fmoc-Lys(Boc)-H($CDCl_3$ δ 9.58 (C**H**O))[26] and Fmoc-Leu-H ($CDCl_3$ δ 9.58 (C**H**O))[29] were synthesized following the same procedure as above.

### 3.6.4 Peptide Synthesis: General Procedure

Peptides were synthesized on the Fmoc-PAL-PEG-PS resin (200 mg, low-load resin), using the Fmoc synthetic strategy, as shown in Figure 3-10, generally with a 4-fold excess of the Fmoc-protected amino acids. The coupling reagents used were PyBOP, HOBt, and DIEA in a 1:1:2 ratio in 4-fold excess with respect to the resin substitution, using DMF (5 mL) as the solvent. Generally coupling reaction times were 2 h (on an automated peptide synthesizer, CSBio) or until a negative ninhydrin test result was observed (on the CHOIR used for manual peptide synthesis[30]). The Fmoc group was removed from the amino acid with piperidine in DMF (20:80 v/v, 2 x 20 min) followed by washing with DMF before coupling the next amino acid. After

the synthesis the peptides were cleaved from the resin for 2 h with Reagent B (88% TFA, 5% $H_2O$, 5% phenol, and 2% TIPS – triisopropylsilane)[31]. The peptides were filtered from the resin, and the filtrate was diluted with 10% aq acetic acid, extracted with ether, and then the ether was back extracted with 10% aq acetic acid. The combined aqueous layers were lyophilized to dryness to give the crude peptides.

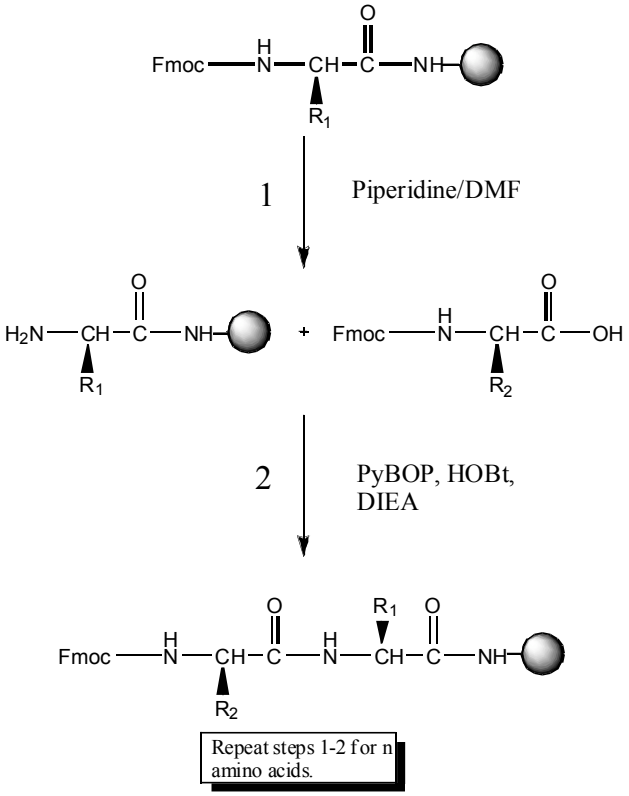


**Figure 3-10: General scheme of peptide synthesis using the Fmoc strategy.**

## 3.6.4.1 HPLC and MS Analysis

The peptides were purified using preparative reversed-phase high performance liquid chromatography (RP-HPLC) (Shimadzu SPD 10AVP HPLC system equipped

with a Shimadzu SPD-10AVP detector) on a Vydac $C_{18}$ column (10 μ, 300 Å, 22 x 250 mm) equipped with a guard cartridge. A linear gradient of 15-50% (or 10-45%) aqueous MeCN containing 0.1% TFA (20 mL/min) over 45-60 min was generally used, and elution was monitored at 214 nm. Purity was evaluated using an analytical HPLC (Shimadzu SPD-10AVP equipped with a Shimadzu SPD-10AVP detector) and a Vydac $C_{18}$ column (5 μ, 300 Å, 5 x 50 mm) equipped with a guard cartridge. The purity was verified using two orthogonal solvent systems - generally 5-50% aqueous MeCN containing 0.1% TFA (1 mL/min) over 45 minutes and 5-50% aqueous MeOH containing 0.1% TFA (1 mL/min) over 45 minutes. Exceptions are noted under the synthesis of particular peptides. The eluents were monitored at 214 nm.

The molecular weights of the peptides were determined by ESI-MS on Waters Q-TOF analyzer.

### 3.6.5 Synthesis of [Leu$^{12}$], [Leu$^{12}$,Leu$^{13}$] and [Lys$^{6}$]arodyn

These peptides were synthezised using the general procedure described above on the CSBio. Acetylation (using 1:1 acetic anhydride/DMF) was performed on the CSBio after the final Fmoc deprotection.

After lyophilization, the crude peptides were purified via preparative HPLC. Pure compounds were analyzed with MS and HPLC, as described above. For [Leu$^{12}$]arodyn the second system used was 20-60% aqueous MeOH (0.1% TFA) over 45 min with a flow rate of 1 mL/min. Pure compounds isolated were [Leu$^{12}$]arodyn: 20 mg, [Leu$^{12}$,Lys$^{13}$]arodyn: 31 mg, and [Lys$^{6}$]arodyn: 10 mg (all >98% purity in both systems).

### 3.6.6 NMeArg Analogs

These peptides were synthesized primarily using the general peptide synthesis techniques described above, on the CSBio and on the CHOIR. Fmoc-NMeArg(Mtr) (in a 2-fold excess) was coupled to the peptide using the coupling reagents PyBOP, HOBt and DIEA (2:2:4 fold excess relative to the resin substitution). The next amino acid in the sequence was coupled using PyAOP, HOAt and DIEA (4:4:8 fold excess). The peptides were cleaved from the resin by mixing with 5 mL of modified Reagent K[18] (90% TFA, 5% thioanisole, 2.5% phenol and 2.5% 3,6-dioxa-1,8-octanedithiol)[19] overnight. A general extraction procedure was followed as described above.

After lyophilization, the crude peptides were purified via preparative HPLC. For [NMeArg$^{9}$]arodyn, the second system used was aqueous MeOH (0.1% TFA) system with a gradient of 20-70% MeOH over 50 min and a flow rate of 1 mL/min. Pure compounds isolated were [NMeArg$^{4}$]arodyn: 12 mg, [NMeArg$^{6}$]arodyn: 12 mg, [NMeArg$^{7}$]arodyn: 9 mg and [NMeArg$^{9}$]arodyn: 8 mg (each >98% purity in two systems).

### 3.6.7 Reduced Amide Bond Analogs

These peptides were assembled partly on the CSBio and partly on the CHOIR. The reduced amide bond was formed by first removing the Fmoc group from the peptide and then the aldehyde (0.5 mmol, 15 equiv) dissolved in DMF solution was added, followed by $NaCNBH_3$ (20 mg, 0.3 mmol, 10 equiv) after 5 minutes. The reaction was bubbled for 3-5 h. After the final Fmoc deprotection, acetylimidazole

(92 mg, 20 equiv) and DIEA (2 equiv) in DCM/DMF (1:2, 5 mL) were added. The reaction was then bubbled for 3 h. The peptide was cleaved from the resin by mixing with Reagent B (5 mL) for 3 h. The extraction procedure was followed as described above.

After lyophilization, the crude peptides were purified via preparative HPLC. Purity was confirmed with the HPLC solvent systems described earlier. Pure compounds isolated were [Phe$^3\Psi$(CH$_2$NH)Arg$^4$]arodyn: 13 mg, [Leu$^5\Psi$(CH$_2$NH)Arg$^6$]arodyn: 6 mg, [Lys$^6\Psi$(CH$_2$NH)Arg$^7$]arodyn: 6 mg and [D-Ala$^8\Psi$(CH$_2$NH)Arg$^9$]arodyn: 10 mg (each >98% purity in two systems).

### 3.6.8 Metabolism Studies

The procedure was followed as described by Reed *et al.*[20] Rat brains were placed in a matrix and sliced; the striatum was dissected out from a 2 mm slice, while on ice, and then the striatal slices were placed on dry ice. After warming to 4ºC on ice, the slices were washed once with phosphate buffered saline (PBS). Additional PBS (100 μL) was added to the rat brain slice for 4 minutes. This solution was then divided into 20 μL portions for each experiment. Two peptides per rat brain were analyzed (with no inhibitors and with bestatin) in addition to a blank control sample. These vials were kept at 4 °C. Bestatin, an aminopeptidase inhibitor, was added to one vial per peptide (1 μL of 10 mM solution for a final concentration of 500 μM). Peptide (2 μL of 100 μM solution for a final concentration of 10 μM) was added to each vial. The vials were then warmed to 37 °C. At each time point (3, 6, 10, 15, 30 and 60 min) 2

μL was removed from each vial, cooled to 4 °C and internal standard was added (10 μM in MeCN, 2 μL, final concentration of 5 μM). The internal standard was a known peptide ([$N^{\alpha}$-benzyl-$Tyr^{1}$,$NMeArg^{7}$]Dyn A-(1-11)$NH_2$, MW 1465)[8].

### 3.6.9 MALDI Analysis

The aliquots (1 μL), already mixed with internal standard as described above, were mixed with the matrix (1 μL), and the mixture was spotted onto the MALDI plate and dried before the analysis. MALDI was performed in the positive ion mode. Instrument: Voyager-DE MALDI STR Biospectrometry Workstation, Applied Biosystems, Foster City. Matrix: α-cyano-4-hydroxycinnamic acid (CHCA). Internal standard: [$N^{\alpha}$-benzyl-$Tyr^{1}$,$NMeArg^{7}$]Dyn A-(1-11)$NH_2$.

### 3.6.10 Binding Studies

Radioligand binding assays were performed using cloned rat κ opioid receptors stably expressed on CHO cells.[32] [$^3$H]Diprenorphine was used as a radioligand in the assays. Nonspecific binding was determined in the presence of 10 μM unlabeled Dyn A-(1-13)$NH_2$. Binding assays were carried out under standard conditions. $IC_{50}$ values were determined by nonlinear regression analysis to fit a logistic equation to the competition data using GraphPad Prism software (GraphPad Software Co., San Diego, CA). $K_i$ values were calculated from the $IC_{50}$ values by the Cheng and Prusoff equation,[33] using $K_D$ value of 0.45 for [$^3$H]diprenorphine. The results presented (Table 3-3) are the mean ± SEM from three separate assays.

## 3.7 References


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# 4 Conclusions and Future Studies

## 4.1 Summary

The objective of this research was to synthesize new arodyn analogs designed to have increased metabolic stability. To meet this objective, 11 analogs were synthesized and 7 of these analogs were tested in metabolism studies. Two extended analogs were prepared to investigate the effect of peptide length on the break down of arodyn. A series of N-methylarginine analogs and a series of reduced amide bond analogs were also synthesized. These backbone modifications preserved the side chain functionalities of arodyn, while stabilizing the bonds that had previously been identified as potential sites of cleavage. One extended analog, the reduced amide bond series and two N-methyl arginine analogs were investigated in the initial metabolism studies.

### 4.1.1 Metabolism

Dynorphin A (Dyn A - H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH) is the most studied endogenous ligand for the kappa opioid receptor (KOR).

Dynorphin A is metabolized by both exopeptidases and endopeptidases. Endopeptidases selective for dibasic and monobasic sites in Dyn A have previously been reported.[1-5] Thimet-oligopeptidase (E.C.3.4.24.15) is a metallo-endopeptidase that has been reported to be involved in the metabolism of a variety of peptides at the Leu-Arg bond, including the dynorphins.[6] Dynorphin A converting enzyme (EC

3.4.22) can cleave at Leu$^{5}$-Arg$^{6}$ and Arg$^{6}$-Arg$^{7}$ as well as at other monobasic sites, and Ile$^{8}$-Arg$^{9}$ is cleaved by Dyn A processing enzyme.[4]

Arodyn, (Ac[Phe$^{1,2,3}$,Arg$^{4}$,D-Ala$^{8}$]Dyn A-(1-11) amide) was developed in the Aldrich laboratory as a dynorphin A analog. Arodyn exhibits high KOR selectivity and antagonist activity at KOR.[7] In conditioned place preference assays in mice, arodyn showed promise as a treatment for stress-induced relapse of cocaine-seeking behavior.[8] However, studies in rat brain slices showed that arodyn is rapidly metabolized, with complete disappearance of the parent compound in under 15 minutes.[9] These studies identified the possible sites of metabolism as at the N-termini of every arginine residue in the arodyn sequence, at internal monobasic and dibasic sites similar to the dynorphin A cleavage patterns. Arodyn is cleaved between residues 3-4 (a monobasic site next to an arginine), and, like Dyn A, at the 5-6, 6-7 and 8-9 peptide bonds in its sequence.

## *4.2 Conclusions*

Replacement of the 3-4, 5-6 or 6-7 peptide bonds with reduced amide bonds produced analogs with increased stability over arodyn – these compounds survived up to 30 to 60 minutes. N-Methylarginine stabilization of the 5-6 or 6-7 peptide bonds did not produce equally stable compounds. Neither [NMeArg$^{6}$]arodyn nor [NMeArg$^{7}$]arodyn survived beyond 15 minutes in the metabolism studies. This could be due to a change in charge in the peptide backbone (the reduced amide bond adds a positive charge) or altered conformation of the peptide backbone from the reduced

amide bond – either of which could alter peptidase binding. Binding studies are in progress for all analogs to determine if KOR binding has been affected. N-Methylarginine and extended analogs show somewhat reduced binding to KOR compared to arodyn's binding, although these all still show affinity in the nanomolar range.

These metabolism studies also identified the $Pro^{10}$-$Lys^{11}$ bond as a frequent site of cleavage. Following these studies, the arodyn metabolism data was reexamined and the $Pro^{10}$-$Lys^{11}$ bond was found to be cleaved. The enzymes cleaving arodyn and these analogs appear to be selective for either arginine or lysine residues.

## *4.3 Future Work*

Future work on this project includes completing the metabolism studies on the remaining analogs. The results from these, and the initial metabolism studies can be used in the design of future arodyn analogs. New arodyn analogs may be designed that include multiple sites of modification. Stabilization of the 3-4 and 5-6 bonds with reduced amide bonds produced analogs with detectable parent compound at 30 and 60 minutes, respectively. These sites also were frequently cleaved in the analogs studied, making these good targets for stabilization in new analogs. In addition, future work may include quantitative studies to determine half-lives for some of the analogs.

Binding studies will be completed on the remaining reduced amide bond analogs. The results of these studies will help direct the design of future analogs.

## 4.4 References


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# Appendix 1

Metabolites of arodyn analogs observed following inculcation in washings from rat brain slices with no inhibitors.

| Appendix 2 Analog / parent peptide | Appendix 3 Metabolites | | | | | |
|---|---|---|---|---|---|---|
| | Appendi min | Appendi min | Appendix 0min | Appendi 5min | Append 0min | Appendi 0min |
| Appendix 11 [Leu$^{12}$,Lys$^{13}$] / 1776 | Appendi 777, 1293, 1024, 867 | Appendi 777, 1293, 1024, 867 | Appendix 777, 1293, 1024, 867 | Appendi 777, 1024, 867 | Append 67 | Appendi o peaks[a] |
| Appendix 18 [Phe$^{3}\Psi$(CH$_2$ NH)Arg$^{4}$] / 1521 | Appendi 522, 1394, 914, 757 | Appendi 522, 1394, 914, 757 | Appendix 522, 1394, 914, 757 | Appendi 522, 1394, 914, 757 | Append 522, 914, 757 | Appendi 14, 757 |
| Appendix 25 [Leu$^{5}\Psi$(CH$_2$ NH)Arg$^{6}$] / 1521 | Appendi 522, 1394, 1038 | Appendi 522, 1394, 1038 | Appendix 522, 1394, 1141, 1038 | Appendi 522, 1394, 1141 | Append 522, 1394, 1141 | Appendi 394, 1141, 911 |
| Appendix 32 [Lys$^{6}\Psi$(CH$_2$ NH)Arg$^{7}$] / 1492 | Appendi 493 | Appendi 493, 1367, 884 | Appendix 493, 1367, 1010, 884 | Appendi 493, 1367, 1010, 884 | Append 367, 1010, 884 | Appendi 367, 884 |
| Appendix 39 [D-Ala$^{8}\Psi$(CH$_2$ NH)Arg$^{9}$] / 1521 | Appendi 522 Appendi | Appendi 522 | Appendix 522 | Appendi 522 | Append o peaks[a] | Appendi o peaks[a] |
| Appendix 47 [NMeArg$^{6}$] / 1547 | Appendi 548, 1066, 939 | Appendi 548, 1066, 939 | Appendix 548, 1066, 939 | Appendi 548, 939 | Append 548, 1066, 939 | Appendi o peaks[a] |
| Appendix 54 [NMeArg$^{7}$] / 1547 | Appendi 548 | Appendi 548 | Appendix 548 | Appendi 548, 1066, 939 | Append 066, 939 | Appendi 39 |

[b]Internal standard was visible but no metabolite peaks were observed above the background.

# Appendix 2

All figures are spectra from experiments with the aminopeptidase inhibitor bestatin.

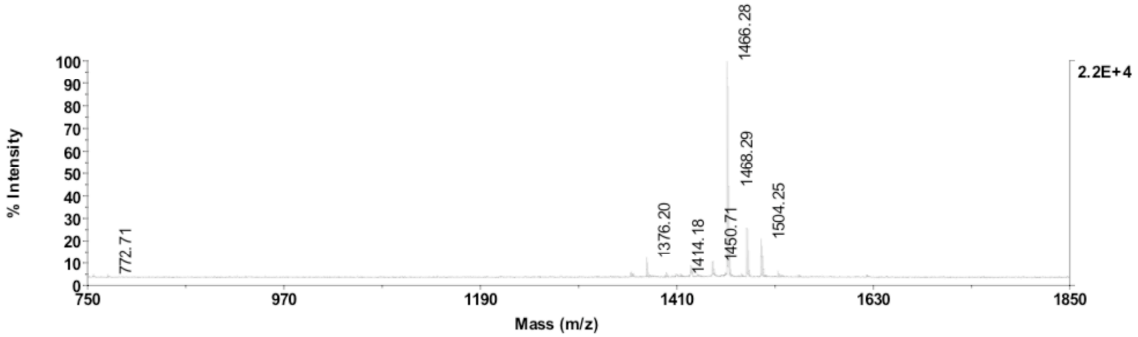


**Figure xi: Blank spectrum of washings from rat brain slices (with internal standard).**

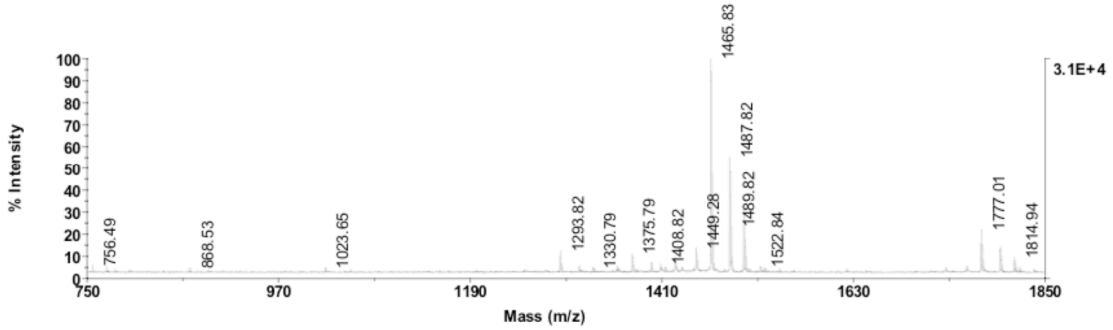


**Figure xii: [Leu$^{12}$,Lys$^{13}$]aroydn metabolism in rat brain slice washing after 3 minutes.**

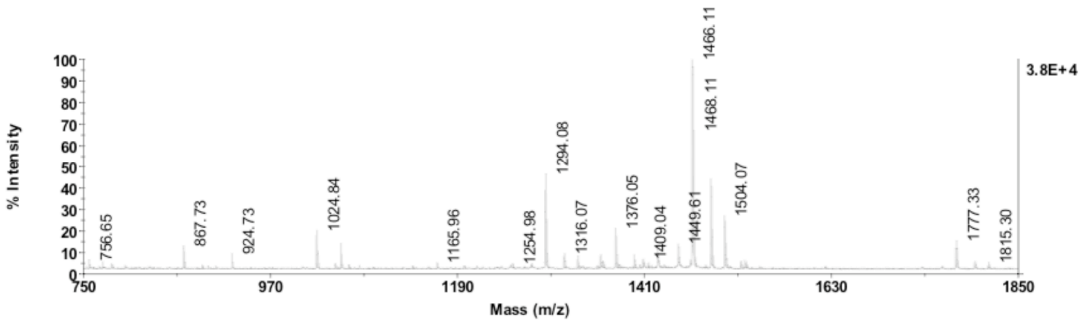


**Figure xiii: [Leu$^{12}$,Lys$^{13}$]aroydn metabolism in rat brain slice washing after 6 minutes.**

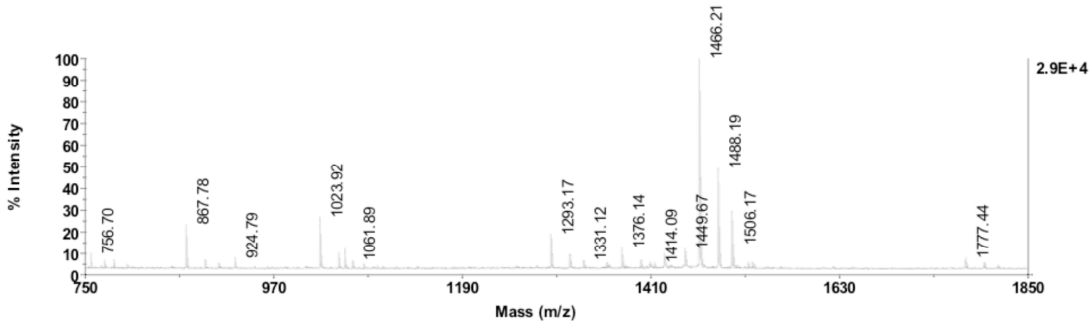


**Figure xiv: [Leu$^{12}$,Lys$^{13}$]arodyn metabolism in rat brain slice washings, 10 minutes.**

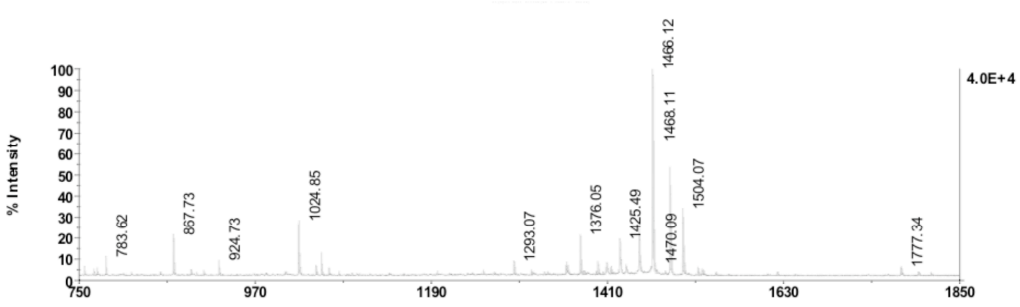


**Figure xv: [Leu$^{12}$,Lys$^{13}$]aroydn metabolism in rat brain slice washing after 15 minutes.**

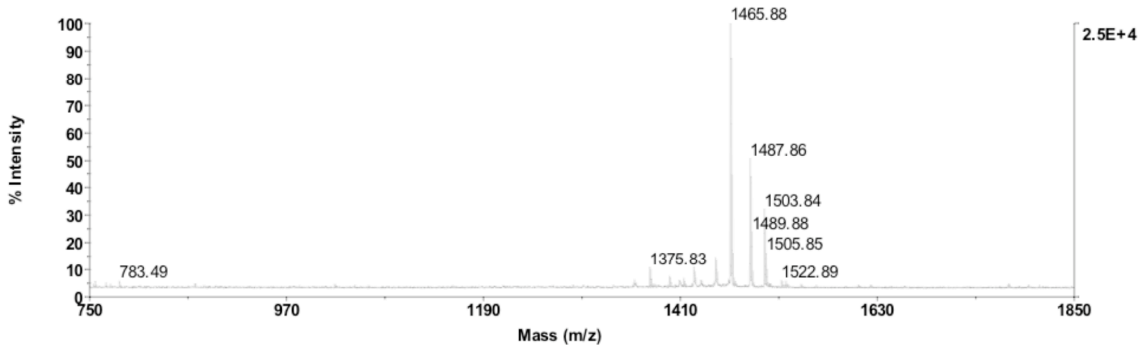


**Figure xvi: [Leu$^{12}$,Lys$^{13}$]aroydn metabolism in rat brain slice washing after 30 minutes.**

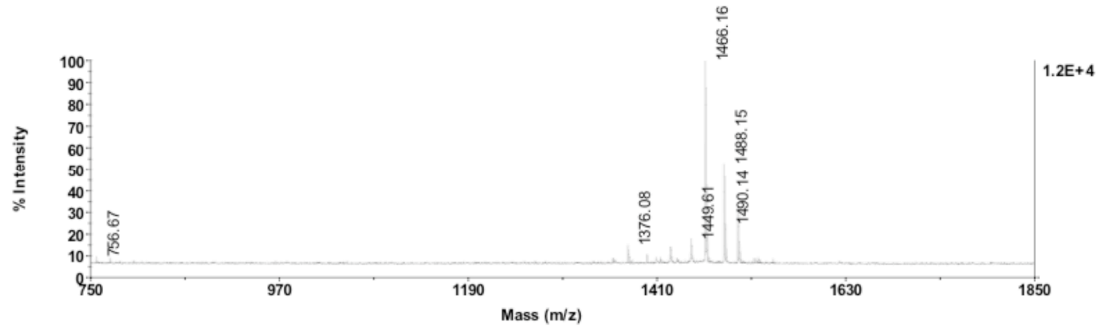


**Figure xvii: [Leu$^{12}$,Lys$^{13}$]arodyn metabolism in rat brain slice washings, 60 minutes (with bestatin).**

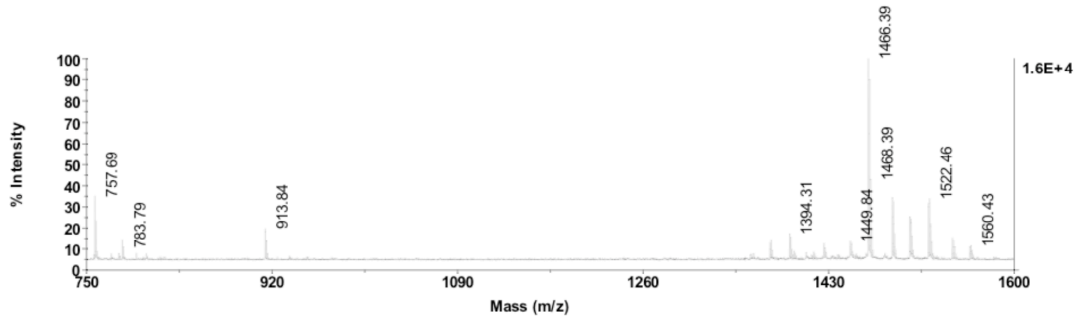


**Figure xviii: [Phe$^{3}$Ψ(CH$_2$NH)Arg$^{4}$]arodyn metabolism in rat brain slice washings after 15 min.**

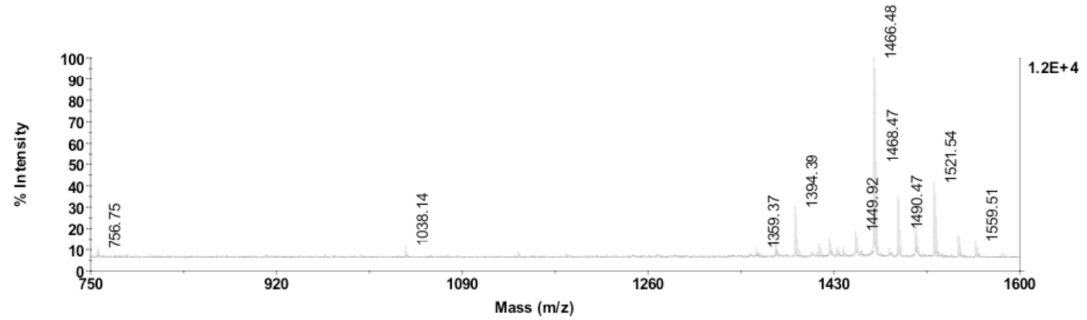


**Figure xix: [Leu$^{5}$Ψ(CH$_2$NH)Arg$^{6}$]arodyn metabolism in rat brain slice washings after 15 min.**

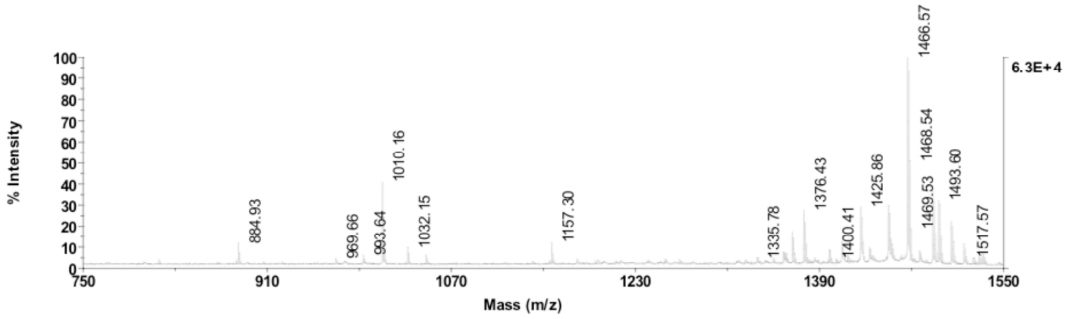


**Figure xx: [Lys$^{6}$Ψ(CH$_2$NH)Arg$^{7}$]arodyn metabolism in rat brain slice washings after 15 min.**

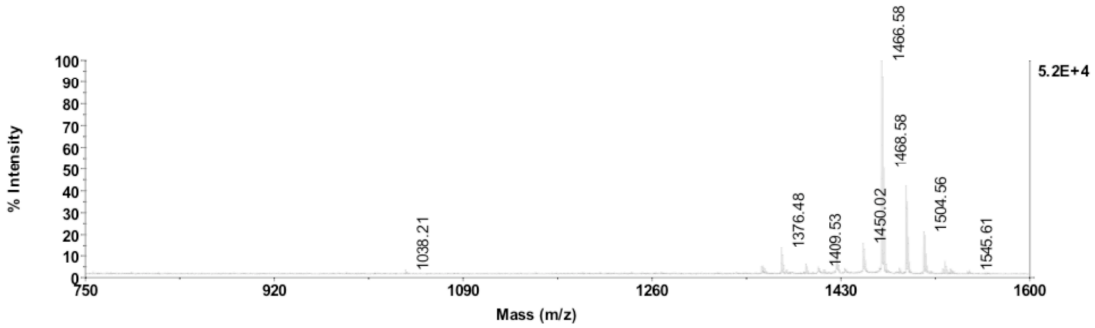


**Figure xxi: [D-Ala$^{8}$Ψ(CH$_2$NH)Arg$^{9}$]arodyn metabolism in rat brain slice washings after 15 min.**

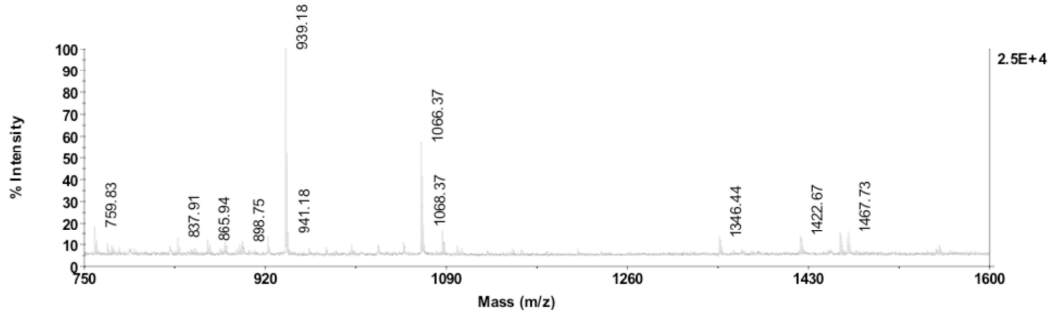


**Figure xxii: [NMeArg$^{6}$]arodyn metabolism in rat brain slice washings after 15 min.**

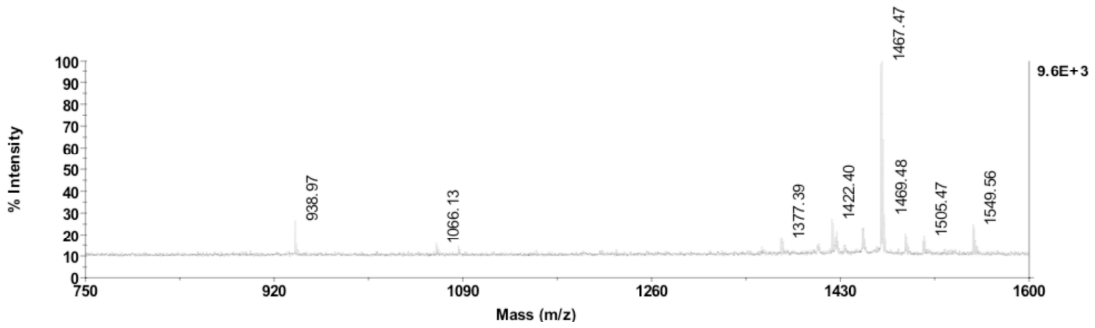


**Figure xxiii: [NMeArg$^{7}$]arodyn metabolism in rat brain slice washings after 15 min.**